Evaluations of Diagnostic Tests for Undifferentiated Febrile Illness on the Thailand-Myanmar (Burma) Border

Thesis

Evaluations of Diagnostic Tests for Undifferentiated Febrile Illness on the Thailand-Myanmar (Burma) Border

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Abstract

Background

Dengue, leptospirosis and rickettsial infections are the most common non-malaria causes of acute undifferentiated febrile illness in Southeast Asia. Current diagnostic tests are inadequate for clinical use.

Methods

We conducted a two year prospective fever study in three outpatient clinics on the Thailand-Myanmar border, recruiting patients aged at least five years with acute undifferentiated febrile illness. The study was divided into two parts: non-malaria and malaria patients. Specimens were tested to determine the causes of fever and to evaluate the clinical diagnostic accuracy of the tests for early diagnosis of dengue, leptospirosis and rickettsial infections.

Results

A total of 1,029 febrile patients were recruited: 908 non-malaria and 121 malaria patients. A laboratory confirmed diagnosis was made in 34.5% of non-malaria patients, of which 15.9% were dengue, 6.0% leptospirosis, 6.0% murine typhus, and 3.2% scrub typhus. Co-infection was found in 1.7% with mostly leptospirosis and scrub typhus (1.5%). In malaria patients, co-infection was found in 8.3% in most cases with scrub typhus (7.4%).

An immunochromatographic test (ICT) (Non-structural protein 1 [NS1] and Immunoglobulin M [IgM]/Immunoglobulin G [IgG] detection) and real-time reverse transcriptase polymerase chain reaction (rRT-PCR) were evaluated for dengue diagnosis using acute plasma specimens. Sensitivities of the ICT and rRT-PCR were 86.1% (95% confidence interval [CI] 79.4-91.3) and 97.2% (95% CI 93.0-99.2), and specificities were

94.9% (95% CI 93.0-96.4) and 99.1% (95% CI 98.1-99.7), respectively compared against IgM/IgG enzyme-linked immunosorbent assay (ELISA). Combining rRT-PCR with ICT improved the sensitivity of the diagnostic process to 98.6% (95% CI 95.1-99.8).

The IgM ICT and 47kDa quantitative real-time PCR (qPCR) for scrub typhus and 17kDa qPCR for murine typhus were evaluated using acute plasma specimens for the IgM ICT and acute buffy coat specimens for the qPCRs. Sensitivities of the IgM ICT, 47kDa qPCR and 17kDa qPCR were 27.3% (95% CI 15.0-42.8), 22.7% (95% CI 11.5-37.8) and 31.5% (95% CI 19.5-45.6) respectively. Specificities of the IgM ICT, 47kDa qPCR and 17kDa qPCR were 93.5% (95% CI 91.5-95.1), 99.6% (95% CI 98.9-99.9) and 99.7% (95% CI 99.0-100), respectively compared to IgM ELISA/indirect immunofluorescence assay (IFA).

A 16S *rRNA* qPCR was validated and implemented for leptospirosis diagnosis. The analytical performance was good, it was found to be 100% specific and had a limit of detection of one copy per microliter (µl) of DNA template.

Sub-microscopic malaria infection detected using 18S *rRNA* qPCR was found in 17.5% of non-malaria patients. The geometric mean of parasitaemia was very low (281.1 parasites/µl [95% CI 172.5-458.2]). It was unlikely to be the cause of fever at this low level.

C-reactive protein (CRP) result was able to distinguish between dengue virus infection (9.0 milligrams per litre (mg/l) (Interquartile range [IQR] 7.9-18.0)) and bacterial infections (106.5 mg/l [IQR 40.8-166.5] for leptospirosis, 55.2 mg/l [IQR 40.7-114.0] for scrub typhus and 24.7 mg/l [IQR 15.0-48.6] for murine typhus, $P<0.0001$ for all).

Conclusion

The new generation ICT that included NS1 antigen detection was clinically useful and appropriate to implement in the field for early diagnosis of acute dengue infection. The rRT-PCR for dengue could replace the gold standard serology for early diagnosis using a single specimen. The IgM ICT, 47kDa and 17kDa qPCRs were inadequate for diagnosis of scrub typhus and murine typhus due to low clinical diagnostic sensitivity. For leptospirosis 16S *rRNA* qPCR, the excellent analytical performance warrants further investigation on its clinical usefulness. The CRP was found to be a useful tool to clinicians for determining whether a patient had a viral or bacterial infection. Early diagnosis of scrub typhus, murine typhus, and leptospirosis remains challenging. Development and clinical evaluation of improved diagnostic tests is urgently needed.

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°C	Degree celsius
°N	Degree north latitude
°S	Degree south latitude
ADE	Antibody-dependent enhancement
ALT	Alanine aminotransferase
AMV-RT	Avian Myeloblastosis Virus reverse transcriptase
ARRL	Australian Rickettsial Reference Laboratory
AST	Aspartate aminotransferase
AUC	Area under ROC curve
AUFI	Acute undifferentiated febrile illness
Bayesian LCMs	Bayesian latent class models
BHQ1	Black Hole Quencher1
BI	Binding index
bp	Base pair
C	Core protein
CAAT	Cross-agglutination absorption test
CBC	Complete blood count
CF	Complement fixation
CFU	Colony forming unit
CI	Confidence interval
CIE	Counterimmunoelectrophoresis
cm	Centimetre

cm^2	Square centimetre
CNS	Central nervous system
CrI	Credible interval
CRF	Case report form
CRP	C-reactive protein
CSF	Cerebrospinal fluid
Ct	Cycle threshold
Cu	Culture
d	Day
DABCYL	4-(dimethylaminoazo) benzene-4-carboxylic acid
DENV	Dengue virus serotype
DF	Dengue fever
DFM	Dark-field microscopy
DHF	Dengue haemorrhagic fever
DMSc	Department of Medical Science
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DSS	Dengue shock symdrome
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
E (or E protein)	Envelope (or Envelope protein)
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immuno assay

ELISA	Enzyme-linked immunosorbent assay
FAM	6-carboxy-fluorescein
FDS	Fever diagnostic study
FITC	Fluorescein isothiocyanate
FUO	Fever of unknown origin
GE	Genomic equivalent
Hct	Haematocrit
HI	Haemagglutination inhibition
hr	Hour
HRP	Horseradish peroxidase
HRP-II	Histidine-rich protein II
ICT	Immunochromatographic test
IFA	Indirect immunofluorescence assay
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHA	Indirect haemagglutination
IIP	Immunoperoxidase
IPD	Inpatient department
IQR	Interquartile range
IU/L	International unit per litre
JE	Japanese encephalitis
Kb	Kilobase

l	litre
LAMP	Loop-mediated isothermal amplification
LB	Luria-Bertani
LERG	Leptospirosis Burden Epidemiology Reference Group
LoD	Lower limit of detection
LOMWRU	Lao Oxford Mahosot Wellcome Trust Research Unit
LPS	Lipopolysaccharide
LVW	*Leptospira* Vanaporn Wuthiekanun
M	Molar
MAT	Microscopic agglutination test
Mb	Megabase
mg	Milligram
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
MIC	Minimum inhibitory concentration
MKT	Mawker Thai
ml	Millilitre
MLA	Mae La
mm^3	Cubic millimetre
mmHg	Millimetre of mercury
MORU	Mahidol-Oxford Tropical Medicine Research Unit
MRC	Mun Ru Chai
n	Number

NEG	Negative extraction control
Nested-PCR	Nested-polymerase chain reaction
Nested-RT PCR	Nested-reverse transcriptase polymerase chain reaction
nm	Nanometre
NPV	Negative predictive value
NS	Non-structural protein
NS1	Non-structural protein 1
NTC	No template control
NTPase	Nucleotide triphosphatase
OD	Optical density
OPD	Outpatient department
OR	Odds ratio
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PCR-REA	Polymerase chain reaction and digestion of chromosomal DNA by restriction endonucleases
PCR-RFLP	Nested-PCR restriction fragment length polymorphism
PFGE	Pulse field gel electrophoresis
PFU	Plaque forming unit
pLDH	Plasmodium lactate dehydrogenase
pmol	Pico mole
PPV	Positive predictive value
prM	Precursor of membrane

PRNT	Plaque reduction neutralisation test
PTB	Pulmonary tuberculosis
PUO	Pyrexia of unknown origin
qPCR	Quantitative real-time polymerase chain reaction
RBC	Red blood cells
RDT	Rapid diagnostic test
RIA	Radioimmunoassay
RMSF	Rocky Mountain spotted fever
RNaseP	Ribonuclease P
RPA	Recombinase polymerase amplification assay
rRNA	Ribosomal ribonucleic acid
rRT-PCR	Real-time reverse transcriptase polymerase chain reaction
RT-LAMP	Real-time reverse transcription-loop-mediated isothermal amplification
RT-PCR	Reverse transcriptase polymerase chain reaction
S	Serology
SE Asia	Southeast Asia
SFG	Spotted fever group rickettsiae
SMRU	Shoklo Malaria Research Unit
SOP	Standard operating procedure
ssRNA	Single stranded ribonucleic acid
STG	Scrub typhus group rickettsiae
STGG	Skim-milk tryptone glucose glycerol

STIC	Scrub typhus infection criteria
T	Temperature
TAMRA	6-carboxytetramethylrhodamine
TET	Tetrachlorofluorescein
TG	Typhus group rickettsiae
Thailand NEQAS	Thailand National External Quality Assurance Scheme
TIA	Thin-layer immunoassay
TMA	Transcription-mediated amplification
TMB	Tetramethylbenzidine
U	Unit
UTI	Urinary tract infection
WBC	White blood cell
WHO	World Health Organization
WPA	Wang Pha
y	Year
µg	Microgram
µl	Microlittre
µm	Micrometre

1 Background and Introduction

1.1 Common causes of acute undifferentiated febrile illness in a rural Southeast Asian setting

Acute undifferentiated febrile illness (AUFI) is the most common clinical presentation to health care services in developing countries and is a major public health problem, especially in rural areas where infrastructure, clinical laboratory facilities and sanitary conditions remain poor [1]. AUFI describes an acute and short duration of febrile illness accompanied by non-specific symptoms and signs, unlike fever of unknown origin (FUO) also known as pyrexia of unknown origin (PUO), which describes prolonged undiagnosed fevers. The definition of AUFI may be distinguished from FUO by fever duration, progression of illness and underlying causes [2, 3].

The causes of fever include a wide variety of infectious and non-infectious diseases such as neoplastic diseases, collagen vascular diseases and numerous miscellaneous diseases, however the majority are caused by infections [3]. The term AUFI has different implications depending on local epidemiology, and often refers to self-limiting viral syndrome or flu-like illness in developed countries. In most developing countries, malaria and other non-malarial infections such as dengue, leptospirosis and rickettsial infections present as AUFI [2, 4]. The actual aetiologies of AUFI are generally overlooked and patients are empirically treated to cover the potential diagnoses based on clinical presentations. Knowledge of local patterns or epidemiology of diseases is crucial for treatment decision-making. The accurate incidence and prevalence of infectious diseases are largely unknown, with estimates based on individual reports and dependent on the interest of local health care providers. There is increasing evidence that most cases of febrile illness in malaria endemic areas are not actually caused by malaria [5]. Data from previous fever studies have confirmed that dengue, leptospirosis, rickettsial infections

(especially scrub typhus and murine typhus), typhoid, chikungunya and Japanese encephalitis (JE) are common causes of non-malaria AUFI in Southeast Asia (SE Asia) including rural areas [6-12]. Many common causes of febrile illness in the tropics have no specific symptoms and signs, with headache, fever and chills being common features [6, 11], making diagnosis in the field difficult, with the exception of malaria, which is easily detectable by microscopy or rapid diagnostic tests [13, 14]. Rapid diagnostic tools are not available for most non-malaria causes of AUFI and those that are available often have poor accuracy and are relatively expensive [15]. Serological diagnostic tools are the standard method for confirmation of these infections, but they require paired acute and convalescent phase specimens, which often are not temporally useful for patient management. New molecular diagnostic tools require an investment in technology, infrastructure and training that is unfeasible in many rural areas. Hence, very few clinical diagnoses are confirmed by laboratory investigation.

1.1.1 Acute undifferentiated febrile illness in SE Asia

The PubMed search term (acute [All Fields] AND undifferentiated [All Fields] AND ("fever" [MeSH Terms] OR "fever" [All Fields])) OR (acute [All Fields] AND undifferentiated [All Fields] AND ("fever" [MeSH Terms] OR "fever" [All Fields] OR "febrile" [All Fields]) AND illness [All Fields]) AND rural [All Fields] AND ("asia, southeastern" [MeSH Terms] OR ("asia" [All Fields] AND "southeastern" [All Fields]) OR "southeastern asia" [All Fields] OR ("southeast" [All Fields] AND "asia" [All Fields]) OR "southeast asia" [All Fields]), in combination with the limiting term for articles published in English, was used to identify relevant studies.

There have been 13 published studies of AUFI conducted in SE Asia including five from Thailand [6, 7, 10, 11, 16], two from Laos [12, 17], three from Cambodia [18-20], one from Vietnam [8], one from Malaysia [21] and one multi-centre study that included

Thailand, Vietnam, Indonesia, Malaysia and Philippines [22]. No studies are available from Myanmar. The causes of AUFI from these studies are summarised in Table 1.1 focussing on dengue, leptospirosis, rickettsial infection and malaria.

These studies were conducted from 1975 to 2011 and included hospital and community based health centres, selecting patients aged from 1-87 years old, and from rural and urban areas. These studies diagnosed a range of different infectious diseases according to laboratory capacity and suspected causes of AUFI. No study used exactly the same laboratory testing as any of the other studies, which makes comparisons difficult. Nevertheless, the results suggested that dengue, leptospirosis, rickettsial infections and malaria account for a significant proportion of AUFI cases in these countries. The proportion of co-infection was variably reported as 0.6-22.8% of cases. Malaria is often co-infected with other pathogens with the highest rate reported when the study included malaria patients diagnosed by nested-polymerase chain reaction (nested-PCR) [18].

In 1984, a study of febrile illness in rural Malaysia reported that scrub typhus was the most frequent diagnosis observed in 19.3% of febrile illness, especially among palm oil labourers, followed by typhoid and paratyphoid fever (7.4%), and flavivirus infection (7.0%). Leptospirosis was found in 6.8% and malaria accounted for 6.2% [21]. In Vietnam, dengue was found to be a major cause of AUFI, responsible for one-third of febrile illness presenting to primary health services [8]. In Laos, the common causes of non-malaria fever were dengue (8.0%), scrub typhus (7.0%), JE (6.0%), leptospirosis (6.0%), and bacteraemia (2.0%) [12]. This is in line with another study from Laos in patients with jaundice and hepatitis who had fever. After excluding hepatitis virus infection, dengue was the most common cause (21.6%), followed by rickettsial infection (8.0%), and leptospirosis (7.9%). Septicaemia accounted for 8.2% of the patients with fever [17].

Table 1.1 Studies of acute undifferentiated febrile illness (AUFI) from SE Asia (focus on dengue, leptospirosis, rickettsial infection and malaria).

1st author Country	Location (position in country)	Year of study	Inclusion criteria	Total patients analysed [n] and age range	Laboratory diagnosis n (%)	Dengue n (%)	Leptospirosis n (%)	Rickettsiosis n (%)	Malaria n (%)	Co-infection n (%)
Capeding **Multicentre** [22]	5 SE Asia countries[a]	Jun-10 to Jul-11	T[1] ≥38°C for ≥2d	289 2-14y	165 (57.0)[b]	33 (11.4)	3 (1.0%)[c]	17 (5.9)	Not tested	Not reported
Chheng **Cambodia** [19]	Siem Reap (NW)	Oct-09 to Oct-10	T[1] ≥38°C <48hr	1,225 <16y	575 (46.9)	198 (16.2)	17 (1.4)	134 (10.9)	24 (2.0)	131/575 (22.8)[d]
Mueller **Cambodia** [18]	Soun Kouma, Ou Chra (W), Snoul (E)	Jan-08 to Dec-10	T[2] >38°C History fever <8d	1,193 7-49y	874 (73.3)[e]	75 (6.3)	112 (9.4)	49 (4.1)	676 (56.7)[e]	146 (12.2)
Mayxay **Laos** [12]	Luang Namtha (NW), Salavan (S)	May-08 to Dec-10	T[2] ≥38°C History fever ≤8d	1,938 5-49y	799 (41.0)	156/1927 (8.0)	109/1934 (6.0)	141/1938 (7.3)	22/1936 (1.0)	101/1938 (5.0)
Watthanaworawit **Thailand** [16]	Tak (NW)	Apr-08 to Aug-08	T[3] ≥38°C History fever <7d	162 15-63y	98 (60.5)	72 (44.4)	6 (3.7)	16 (9.9)	Excluded	1 (0.6)
Kasper **Cambodia** [20]	9 health care clinics (SC)	Dec-06 to Dec-09	T[2] >38°C for ≥24hr and <10d	9,997 ≥2y	Not defined (38.0)	883/9,975 (8.9)	2,076/9,975 (20.8)[f]	-[g] (32.0)	716/9,954 (7.2)	94 (1.0)[h]
McGready **Thailand** [10]	Tak (NW)	May-04 to Jan-06	T[2] >37.5°C Pregnant	203 Not defined	138 (65.4)[i]	20 (9.5)[i]	5 (2.4)[i]	26 (12.3)[i]	51 (24.2)[i]	8 (3.9)

Table 1.1 Studies of acute undifferentiated febrile illness (AUFI) from SE Asia (focus on dengue, leptospirosis, rickettsial infection and malaria) (continued).

1st author Country	Location (position in country)	Year of study	Inclusion criteria	Total patients analysed [n] and age range	Laboratory diagnosis n (%)	Dengue n (%)	Leptospirosis n (%)	Rickettsiosis n (%)	Malaria n (%)	Co-infection n (%)
Syhavong **Laos** [17]	Vientiane (S)	May-01 to May-04	Jaundice/↑AST/ALT	392 0.4-83y	130/392 (33.2) excluding Hepatitis A	30/139 (21.6)	24/303 (7.9)	24/301 (8.0)	0/68 (0)	44/392 (11.2)
Phuong **Vietnam** [8]	Binh Thuan (S)	Apr-01 to Mar-02	T[1] ≥38°C and history fever <14d	697 4-82y	234 (33.6)	45 (6.5)[j] 234 (33.6)	9 (1.3)[j]	Not reported	Excluded	Not reported
Suttinont **Thailand** [7]	5 centres (NE, S, C)[k]	Jul-01 to Jun-02	History fever <15d	845 15-87y	577 (68.3)	64 (7.6)[l]	312 (36.9)	206 (24.4)	Excluded	112 (13.2)
Ellis **Thailand** [11]	Sangkhlaburi (W)	Jun-99 to Mar-02	T[3] ≥38°C or history fever <48hr	613 20-87y	294 (48.0)	9 (1.5)	107 (17.5)	36 (5.9)	155 (25.3)	34 (5.5)
Leelarasamee **Thailand** [6]	10 community based hospitals (N, NE, C, E, S)	1991-1993	T[4] >38.3°C in <24hr and History fever 3-14d	1,137 >2y	471 (38.7)	70 (5.7)	14 (1.1)	156 (12.8)	Excluded	65 (5.4)
Brown **Malaysia** [21]	Mentekab (C)	1975-1979	Not reported	1,629 Not defined	1,025 (62.9)	114 (7.0)[m]	110 (6.8)	327 (20.1)	101 (6.2)	14 (0.9)

Remark: ALT=Alanine aminotransferase, AST=Aspartate aminotransferase, °C=Degree celsius, d=Days, hr=Hours, n=number, position in country: (C=Central, E=East, N=North, S=South, W=West), T=Temperature ([1]Axillary, [2]Tympanic, [3]Not specified, [4]Oral), y=Years

[a]Indonesia, Malaysia, Philippines, Thailand, Vietnam; [b]IgM positive for at least one of dengue, chikungunya, hepatitis A, influenza A, leptospirosis, rickettsia, and *S. typhi*; [c]Detected, but not confirmed; [d]Number of co-infections in microbiologically positive episodes; [e]Polymerase chain reaction (PCR) based diagnosis of malaria; [f]Serologically identified by IgM Enzyme−linked immunosorbent assay (ELISA), but not confirmed by Microscopic agglutination test (MAT); [g]Scrub typhus, typhus group and spotted fever group IgG tested positive on convalescent specimen 133/1,906 (7.0%), 261/1,946 (13.4%), and 146/1,263 (11.6%), respectively, and then confirmed on paired specimens 35/133 (26.3%), 56/261 (21.5%), and 21/146 (14.4%), respectively; [h]Active co-infection by a combination of PCR, blood culture, and/or malaria smear; [i]Number of diagnoses (n=211) in pregnant woman (n=203) with fever was shown; [j]Presumptive diagnosis; [k]Nakhon Rachasima, Loei, Bureeum (NE), Chumphon (S), Ratchaburi (C); [l]Obvious dengue cases were excluded; [m]Flavivirus.

In Cambodia, the biggest study and the first systemic assessment of the aetiologies of AUFI was conducted between 2006 and 2009 in the south central part of the country. Influenza viruses were reported as the most frequent pathogens (19.9%), followed by dengue (8.9%), malaria (7.2%) and bacterial infection (6.3%) [20]. A subsequent prospective study of AUFI in rural Cambodia showed that malaria was the most frequent cause (56.7%). Leptospirosis was the second most common observed in 9.4%, followed by dengue (6.3%), and rickettsiosis (4.1%) [18]. Another prospective study in hospitalised children in Northwest Cambodia reported dengue (16.2%), scrub typhus (7.8%), and JE (5.8%) as the most common causes of AUFI in this population. Invasive bacterial infection accounted for 6.3% of the febrile episodes with *Salmonella enterica* serovar Typhi responsible for the majority of infections (1.8% of febrile episodes) [19]. There were similar findings from a multi-centre study which reported that chikungunya, typhoid, and dengue were the most common identified causes of children with AUFI in SE Asia [22].

The rates of dengue, leptospirosis and rickettsial illness are not directly comparable between studies (Table 1.1). Firstly, the enrolment of patients to these studies was highly inconsistent. Temperature was measured by different methods: four studies used tympanic [10, 12, 18, 20], three studies used axillary [8, 19, 22] and one study used oral temperature [6]. There were three studies that did not specify how temperature measurement was done for inclusion although a cut-off point for fever was defined [11, 16, 17]. One further study did not require a measured temperature for inclusion only specifying days of fever [7] and in the study from Malaysia patients were reported to be febrile but no cut-off point or temperature measurement method was reported [21]. Secondly, history of fever was similarly variably defined from less than 24 hours to less than 15 days of fever or not described at all. Thirdly, the age, sex and pregnancy status of enrolled patients varied. Finally, different laboratory methods and diagnostic algorithms were used for confirmation of diseases.

1.1.2 Acute undifferentiated febrile illness in Thailand

In Thailand, the terminology of acute pyrexia of unknown origin (acute PUO) is used in the reporting system. It is one of the communicable diseases that must be reported to the Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health. It was suggested that the term acute PUO in the Thai reporting system should be changed to acute undifferentiated febrile illness (AUFI) due to the fact that it does not conform with the definition of classical PUO [3, 6]. In this thesis, the term AUFI will be used instead of acute PUO.

Based on Thai surveillance data there were around 200,000-500,000 cases of AUFI reported each year between 2003 and 2014, with an annual morbidity rate ranging from 294.38 to 807.00 per 100,000 population. The number of cases and morbidity rate were highest in 2013 (Figure 1.1). During the same period of time, the trend in the number of deaths and mortality rate was opposite to that for the number of cases and morbidity incidence rate. The number of deaths was highest in 2003 and has declined since then, with the mortality rate now below 0.05 per 100,000 population (Figure 1.2). However, these numbers are likely to be unreliable as they are based on clinical diagnosis alone, and rarely confirmed by the laboratory. It is also difficult to obtain an accurate estimate of mortality when convalescent sera for laboratory confirmed diagnosis are not available from most fatal cases.

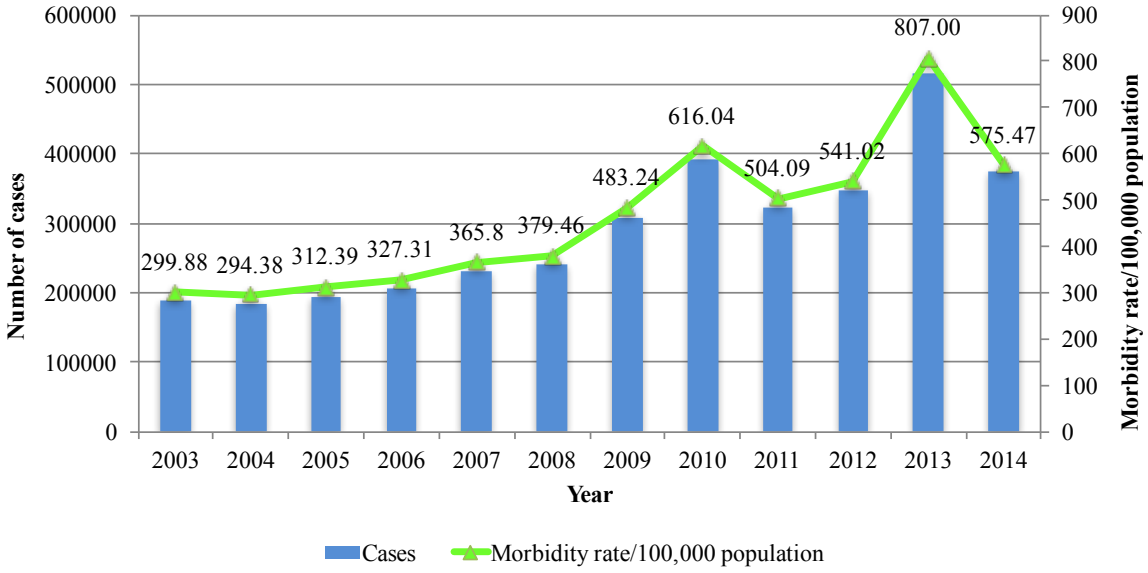

Figure 1.1 Number of cases and morbidity rate of AUFI reported in Thailand between 2003 and 2014.

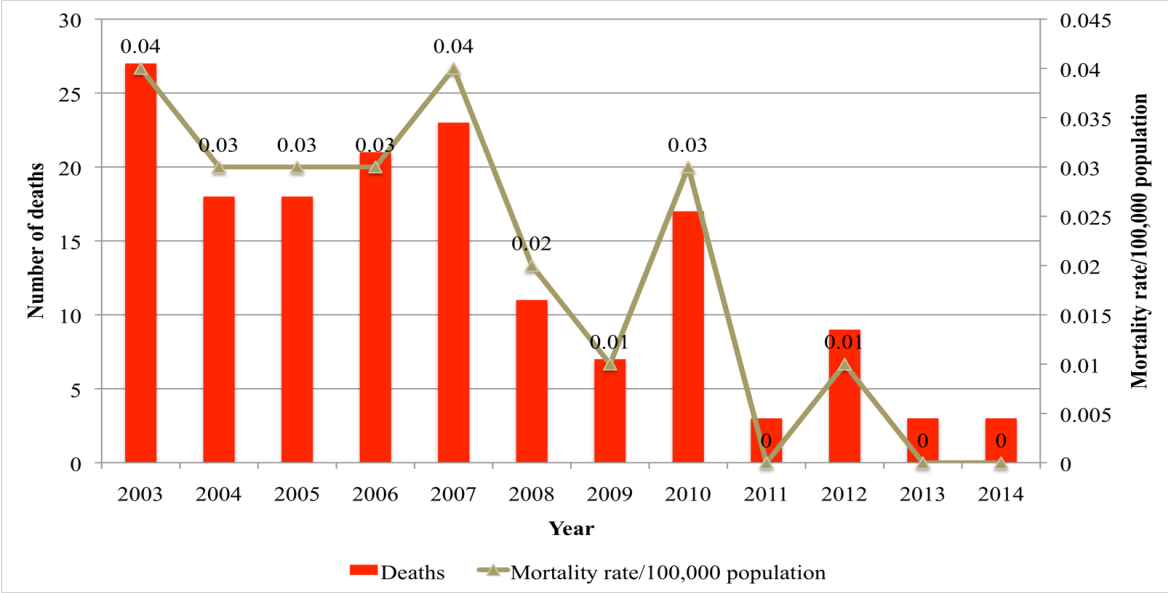

Figure 1.2 Number of deaths and mortality rate of AUFI reported in Thailand between 2003 and 2014.

Source for Figures 1.1 and 1.2: Centre of Epidemiological Information, Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand, http://www.boe.moph.go.th/boedb/surdata/disease.php?dcontent=old&ds=18.

When a country has a strong malaria control program, such as Thailand where patients can access a rapid and accurate diagnosis of malaria, physicians are often faced with the problem of AUFI. The more malaria is brought under control the more the relative frequency of AUFI patients becomes apparent [23]. This is one likely explanation for Thailand recognising AUFI as a significant problem and conducting some of the earliest AUFI studies more than 20 years ago. Figure 1.3 maps the locations of study sites of AUFI in Thailand.

The first was an epidemiological study (Leelarasamee *et al.,* 2004) conducted in 10 community-based hospitals that represented each part of Thailand (North, Northeast, Central, East and South) [6]. The specific location of the study sites was not mentioned, hence they are not in Figure 1.3. Hospitals enrolled patients over a one year period between 1991-1993 and inclusion criteria were outpatient presentation of patients aged over two years with a 3-14 day duration of fever and an oral temperature over 38.3°C, with no obvious single organ involvement or infectious disease that could be diagnosed by physical examination, as well as negative or non-conclusive results of complete blood count, thick film for malaria (i.e. malaria patients were excluded from this study), urinalysis and chest x-ray. This study showed that the most common causes of AUFI were rickettsial infections, influenza, and dengue fever, with scrub typhus found in 7.5%, followed by influenza (6.0%), dengue fever (5.7%), murine typhus (5.3%), enteric fever (1.9%), chikungunya infection (1.1%), leptospirosis (1.1%) and melioidosis (0.9%). Primary bacteraemia was found in 3.2% by blood culture with *Escherichia coli*, streptococci, salmonella, *Enterobacter* spp., and *Staphylococcus aureus* being the five most common pathogens. Co-infection (two or more pathogens identified) was found in 5.4% of the patients. The majority of patients (61.3%) remained undiagnosed.

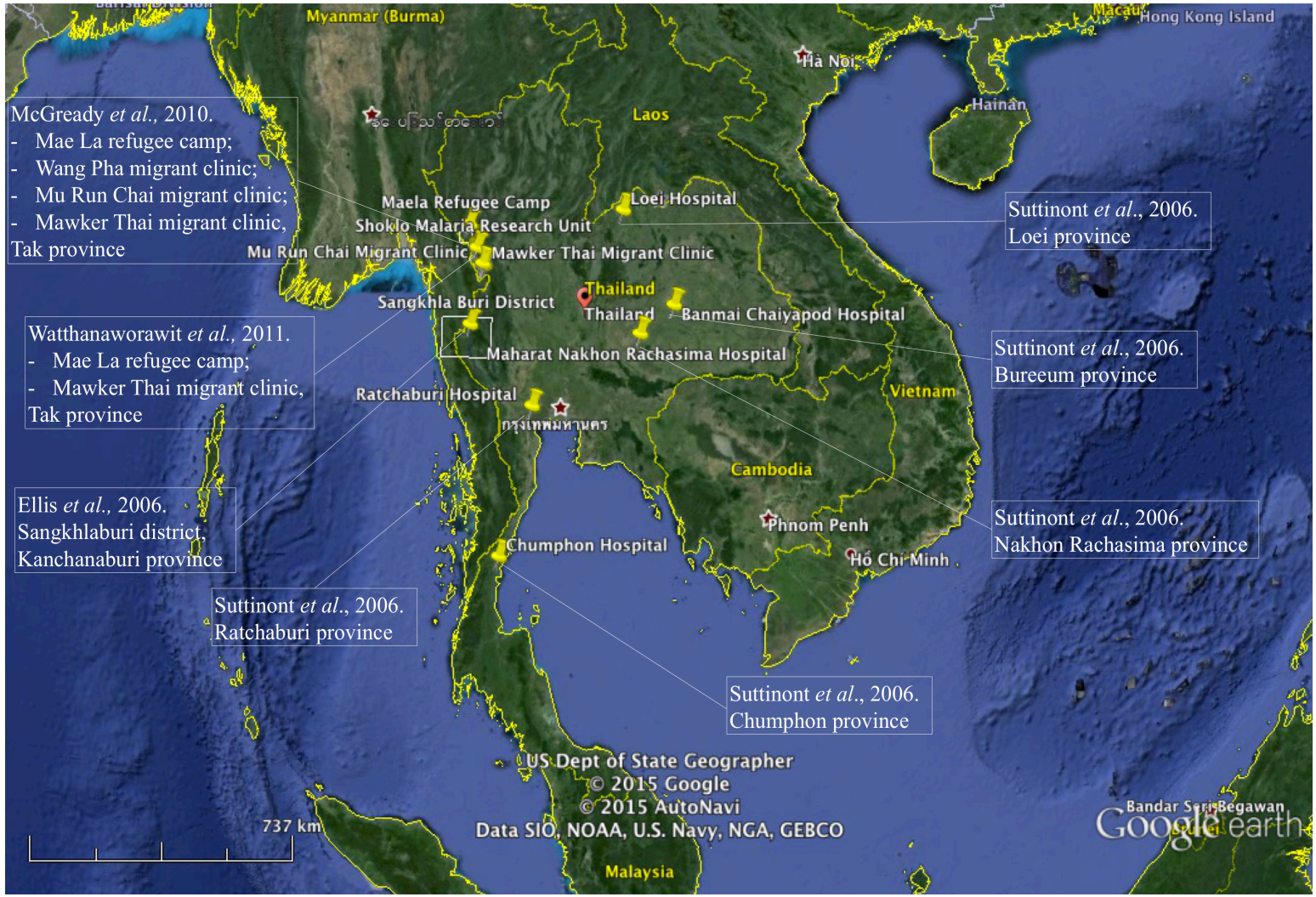

Figure 1.3 A map showing the locations of study sites of AUFI in Thailand [7, 10, 11, 16].

The second published study (Ellis *et al*., 2006) was conducted from June 1999 to March 2002 in the rural area of the west border of Thailand in Sangkhlaburi district, Kanchanaburi province on the border with Myanmar (Figure 1.3), which is home to a variety of ethnic groups including Thai, Karen, Mon, and Burman [11]. In this study, the inclusion criteria were adult inpatients and outpatients presenting with a temperature ≥38°C or history of fever over the previous 48 hours (and patients with fever longer than 48 hours as long as the cause of fever was not yet established). Malaria patients were included in the study and this was found to be the most common cause (25.3%), followed by leptospirosis (17.5%), rickettsial infections (5.9%; with 3.3% spotted fever group (SFG), 1.5% murine typhus and 1.1% scrub typhus), dengue fever (1.5%), and several cases of JE, AIDS, typhoid, and pulmonary tuberculosis (PTB). This was in contrast with the study of Leelarasamee *et al.,* 2004 where rickettsial infection, mostly scrub typhus, was the major cause whereas the major rickettsiosis observed in this study was SFG, which has been rarely reported in Thailand. Co-infections were common (5.5%), particularly for malaria and leptospirosis. No specific diagnosis was confirmed in 52% of the patients.

The third study (Suttinont *et al*., 2006) also included adult patients from five different hospitals including three in the Northeast (Maharat Nakhon Rachasima Hospital in Nakhon Rachasima province, Loei Hospital in Loei province, and Banmai Chaiyapod Hospital in Bureeum province), one in the South (Chumphon Hospital in Chumphon province), and one in the centre of the country (Ratchaburi Hospital in Ratchaburi province) (Figure 1.3), recruited over a one year period between July 2001 and June 2002 [7]. Patients presenting with acute fever of less than 15 days duration and without an obvious focus of infection were included and those with malaria or clinically obvious dengue virus infection were excluded. From this study, leptospirosis was the major cause (36.9%) of adult AUFI and scrub typhus (19.9%) was the second most common disease found, followed by viral infection including dengue and influenza (10.7%), murine typhus

(2.8%), *Rickettsia helvetica* (1.3%), Q fever (1.0%), and other bacterial infections (1.2%). Thirteen percent of the patients had co-infections. The most common was co-infection with leptospirosis and scrub typhus (3.9%), followed by scrub typhus and influenza co-infection (3.4%). The causes of AUFI remained unknown in almost one third of cases.

The fourth published study (McGready *et al.,* 2010) was from the rural area of Tak province on the Thailand-Myanmar border, more than 500km north west of Bangkok, and was specifically conducted in pregnant women. The study included refugee and migrant women of Karen and Burman ethnic groups living and working on both sides of the border [10]. This study was conducted from May 2004 to January 2006 at Shoklo Malaria Research Unit (SMRU) clinics located in Mae La (MLA) refugee camp, and at three sites for migrants named after their village locations in Thailand: Wang Pha (WPA), Mun Ru Chai (MRC) and Mawker Thai (MKT). The inclusion criteria for this study were pregnancy, regardless of gestation, agreed to hospitalisation and a confirmed temperature of >37.5°C. The exclusion criterion was enrolment in a *Plasmodium falciparum* malaria study that was being conducted concurrently. This resulted in the study being biased towards including febrile cases of *Plasmodium vivax* malaria. Despite this malaria was the leading cause (24.2%) of fever, followed by pyelonephritis (19.4%), rickettsial infection (12.3%), dengue (9.5%), acute respiratory infection (8.1%), and leptospirosis (2.4%). Several cases of other causes were observed including enteric fever, suspected chorioamnionitis, endometritis, chicken pox, cholecystitis, gastroenteritis, pyelolithiasis and asymptomatic urinary tract infection. Co-infection was found in 3.9% of the patients, mainly malaria and rickettsia. Causes of AUFI remained unknown in 19.0% (40/211), however, if malaria was excluded the proportion of unknown causes increased to 25.0% (40/160).

The final study was a pilot study (Watthanaworawit *et al.,* 2011) conducted prior to the work described in this thesis and excluded patients with malaria [16]. This study was conducted from April to August 2008 at two of the SMRU clinics: MLA and MKT (Figure 1.3). The inclusion criteria were adults (defined as those aged ≥15 years) with a temperature of ≥38°C and of less than seven days duration. The study did use clinical symptoms or physical signs consistent with dengue, leptospirosis, or rickettsiosis (abnormal bleeding, eye redness, headache, myalgia or rash) as an inclusion criterion and excluded those who had a clear alternative diagnosis such as malaria, urinary tract infection (UTI) or pneumonia. Unsurprisingly, dengue was the main pathogen detected (44.4%), followed by murine typhus (5.6%), scrub typhus (4.3%), leptospirosis (3.7%), and typhoid (2.5%). Co-infection was found in only one case (acute secondary dengue infection and typhoid), and 39.5% remained undiagnosed.

1.1.3 Acute undifferentiated febrile illness and mortality

As most AUFI never receives a confirmed laboratory diagnosis, it is difficult to obtain good estimates of mortality. While there are many case reports available these are inherently biased. In addition, studies of AUFI are limited in terms of sample size and resources. Resource limitations reduce the number of laboratory tests that can be performed to determine aetiology and patients who die usually do not have convalescent sera available, resulting in reduced ability to confirm a diagnosis. In the 13 studies in Table 1.1, six did not report on mortality [7, 8, 16, 18, 20, 22]. Mortality data were reported in seven studies: four hospital-based studies (recruited in the inpatient department [IPD]) [10, 17, 19, 21], one community-based study (recruited in the outpatient department [OPD]) [6], and two studies recruiting patients in both the OPD and IPD [11, 12].

In both studies from Laos mortality was reported [12, 17]. In the most recent study (Mayxay *et al.,* 2013) that recruited inpatients and outpatients (n=1,938), there were six

inpatient deaths giving an overall case fatality rate of 0.7% (6/857). Causes of death included one from each of the following: melioidosis, *Staphylococcus aureus* bacteraemia, JE, dengue, no diagnosis and one not detailed [12]. The other study from Laos (Syhavong *et al.,* 2010) recruited patients who were admitted to hospital with a focus on aetiology in patients with jaundice and/or raised aspartate aminotransferase/alanine aminotransferase (AST/ALT). The report included significantly more deaths: 13 died in hospital amongst 389 patients with discharge results, equivalent to an overall case fatality rate of 3.3%. Amongst these deaths the aetiology included hepatitis C virus (2), hepatitis A virus (1), hepatitis B virus (1), leptospirosis (1), scrub typhus (1) and unknown (7) [17].

Mortality was reported in a paediatric hospital-based study from Cambodia. Sixty nine of 1,225 children died or were discharged to die at home (overall case fatality rate 5.6%). The diagnoses included 19 invasive bacterial infections, 19 patients with clinical pneumonia, 11 patients with viral infection, and seven patients with other primary clinical diagnoses such as clinical diarrhoea or UTI. The source of fever was unknown in 13 patients [19].

Three studies from Thailand, including two from the Thailand-Myanmar border, reported mortality; one community-based study [6], one hospital-based study [10], and one study recruiting patients in both IPD and OPD [11]. The study of Leelarasamee and colleagues reported 13 deaths amongst 1,137 patients recruited in OPD (case fatality rate of 1.1%) and most of these were secondary to nosocomial infections following admission for the underlying disease. The aetiology assigned was one case with dengue, influenza and murine typhus, two with *Staphylococcus aureus* bacteraemia and 10 were unknown [6]. In the pregnancy study, of 203 pregnant women who agreed to hospitalisation and were enrolled, one died (overall case fatality rate 0.5%) and no diagnosis was available for her [10]. In West Thailand, eight of 613 patients died, giving an overall case fatality rate of

1.3%; including two typhoid, one leptospirosis, two end-stage HIV infection, two with no specified diagnosis, and one hepatitis death but with no specific aetiology assigned [11].

Finally, in Malaysia, there were eight confirmed deaths in the 1,629 patients but the fate of 47 who left the hospital against medical advice was not available. Hence, the overall case fatality rate was 0.5% (eight of 1,582) [21], although the mortality rates are likely to have been underestimated. There were two patients who died of typhoid, two of septicaemia and one each of pulmonary tuberculosis, melioidosis, scrub typhus and unknown cause.

From these 13 studies reporting on 20,320 patients with AUFI in SE Asia, there were 118 deaths reported from 7 studies [6, 10-12, 17, 19, 21], or an overall case fatality rate of 2.0% (118 of 6,006; 95% confidence interval [CI] 1.6-2.3). The case fatality rates for hospital-based studies, community-based studies and the study that included patients from IPD and OPD were approximately 2.7% (91/3,399; 95% CI 2.2-3.3), 1.1% (13/1,137; 95% CI 0.6-1.9), and 1.0% (14/1,470; 95% CI 0.5-1.6), respectively. Aetiology of death was ascertained for approximately three-quarters of the cases: 70.3% (83/118).

1.1.4 Acute undifferentiated febrile illness and clinical features

Headache, fever and chills are common clinical features of most infectious causes of AUFI. In the Cambodian AUFI study of almost 10,000 outpatients, it was observed that the most common clinical features were headache (69.5%), cough (57.5%), chills (48.1%), malaise (46.8%) and sore throat (46.6%) [20]. In the Lao study of patients aged 5-49 years, few clinical features were useful to predict the aetiology of non-malaria fever. Briefly, it was observed that platelet count of less than 100,000/µl was associated with dengue; female gender and hepatomegaly with scrub typhus; and age less than 15 years, vomiting, cough and c-reactive protein (CRP) more than 5mg/l were associated with leptospirosis [12]. In adults in Thailand, patients with confirmed dengue had lower white cell counts

(4.8 vs 7.2 x10^3/cubic millimetre (mm^3), P<0.0001) and platelets (147 vs. 162 x10^3/mm^3) than patients with non-dengue infection [16], while clinical symptoms did not differ significantly between the two groups. In Vietnam 48.9% (22/45) of clinically diagnosed dengue cases were serologically confirmed. In the same study 32.5% (212/652) of patients not diagnosed as dengue were positive by serology, indicating a very poor agreement between clinical and serological diagnosis [8]. In pregnant women with AUFI in Thailand, physician predicted diagnosis (excluding malaria) was poor: 22.4% (4/18) of dengue, 19.0% (4/21) of rickettsial infections and 16.7% (1/5) of leptospirosis cases were confirmed by laboratory testing [10]. In Laos hospitalised patients with jaundice or raised AST/ALT, those with leptospirosis and typhus had higher total bilirubin:AST ratio and total bilirubin:ALT ratio than patients with dengue, septicaemia or viral hepatitis (A, B, C, and E) [17]. In West Thailand, a regression analysis of 107 cases of leptospirosis and 36 cases of rickettsiosis did not reveal any differential clinical features [11].

1.1.5 Acute undifferentiated febrile illness and diagnostics

The difficulty of diagnosing these infections clinically is compounded by a lack of diagnostic test availability in the disease endemic areas. Hence, very few clinical diagnoses are confirmed by laboratory investigation. In the 13 studies described above, different laboratory tests were used to establish a diagnosis and sometimes further testing was required for confirmation. To comprehend the breadth of testing for AUFI, the dengue, leptospirosis and rickettsial diagnostics and the use of non-specific markers testing (complete blood count (CBC) and CRP) are summarised in Table 1.2. Most studies used serological tests to confirm diagnosis of these infections. Culture of organisms was rare: only one study from Malaysia did cell culture for flaviviruses [21], three studies performed culture for leptospirosis [7, 10, 12] and three for rickettsiosis [10, 12, 21]. Only one study used rapid diagnostic tests for dengue, leptospirosis and scrub typhus in the field with subsequent confirmation by serology and non-structural protein 1 (NS1) antigen detection

for dengue, culture and serology for leptospirosis, and culture, serology and PCR for scrub typhus [10]. In a study from Cambodia, PCR and nucleotide sequencing of all PCR products were used to improve specificity to define the causes of these infections [18]. Blood culture was commonly performed for other bacterial infections and CBC was also common for routine clinical investigation. CRP testing was used in only two recent studies from Laos [12] and Cambodia [18]. Amongst the 13 studies, only the recent study from Laos [12] had diagnostic and confirmation tests of diseases which covered the detection windows of antigen, antibody and organism/nucleic acid and this study also conducted the greatest number of tests of all the studies.

Data from Table 1.2 contains the range of diagnostic tests used in different studies. This table does not illustrate the true complexity of the diagnostic methods used: serology test kits were purchased from different manufacturers; some studies followed manufacturer's instructions while others used in-house methods; quantitative cut-offs for positivity were not consistent between studies in part due to different background endemicity; and diagnostic algorithms were study dependent. For example, in the study of Capeding *et al.* [22], the Platelia Dengue NS1 Ag kit (Bio-Rad, USA), Dengue Virus IgM Capture DxSelect ELISA kit and Dengue Virus IgG Capture DxSelect ELISA kit (Focus Diagnostics, USA) were used to define dengue infection status. The Leptospirosis Indirect Haemagglutination (IHA) test (Focus Diagnostics, USA) was used to detect leptospirosis. The Rickettsia Indirect Immunofluorescence assay (IFA) IgG/IgM (Focus Diagnostics, USA) was used for Rickettsia. These tests were used according to the manufacturer's instructions. In another study from Mayxay *et al*. [12], a combination of four different ELISA kits was used for the detection of dengue and JE (Panbio, Brisband, Australia): one for the detection and differentiation of IgM against dengue and JE (Japanese encephalitis-Dengue IgM Combo), one for detection of high level of anti-dengue IgG in acute secondary infection (Dengue IgG Capture), one for low level of anti-dengue IgG (Dengue

IgG indirect ELISA) and one for detection of NS1 (Dengue Early ELISA). This combination was performed according to the manufacturer's instructions. The microscopic agglutination test (MAT) was used for confirmation of leptospirosis using a cut-off titre of ≥1:400 or four-fold rise in paired sera to define positives. IFA was used for detection of IgG or IgM antibodies against *Orientia tsutsugamushi* and *Rickettsia typhi.* A positive result was defined as ≥1:400 titre of IgM or IgG. The author noted that it was uncertain whether the cut-off titre used in the study was correct for the situation in Laos. Another example is that even using the same technique for laboratory tests, there are some differences in detailed procedure such as in the study of Leelarasamee *et al.*[6], leptospirosis was diagnosed using MAT test which included only *Leptospira bataviae* as representative serogroup and the cut-off titre was 1:100 or more, whereas a panel of reference strains from 24 serogroups of *Leptospira* and the cut-off titre of at least 1:400 or four fold increase in paired specimens were used in the study of Suttinont *et al.* (leptospire culture was also performed) [7].

Despite a large number of studies using serological methods and culture of organisms for these infections, the tests were used for confirmation rather than rapid diagnosis for patient care. This is mainly because serology traditionally requires paired acute and convalescent specimens. Culture takes a long time and needs sophisticated laboratory facilities and technicians. Molecular methods such as PCR have been used for acute diagnosis of infection as well as rapid diagnostic tests (RDT) to assist patient management. However, in the rural areas where the diseases are endemic, even these diagnostic tests are not routinely available.

Table 1.2 Laboratory diagnostics of acute undifferentiated febrile illness (AUFI) used in the studies from mainland SE Asia.

1st author	Dengue				Leptospirosis				Rickettsia				Malaria			Blood culture	CBC	CRP
	S	NS1	RDT	PCR	Cu	S	RDT	PCR	Cu	S	RDT	PCR	Smear	RDT	PCR			
Capeding [22]	✔	✔	-	-	-	✔	-	-	-	✔	-	-	-	-	-	-	✔	-
Chheng [19]	✔	✔	-	-	✔	-	-	✔	-	✔	-	✔	✔	-	-	✔	✔	-
Mueller [18]	-	-	-	✔	-	-	-	✔	-	-	-	✔	✔	✔	✔	✔	-	✔
Mayxay [12]	✔	✔	-	✔	✔	✔	-	✔	✔	✔	-	✔	✔	✔	✔	✔	✔	✔
Watthanaworawit [16]	✔	✔	-	✔	-	✔	-	-	-	✔	-	-	-	-	-	✔	✔	-
Kasper [20]	✔	-	-	✔	-	✔	-	-	-	✔	-	✔	✔	-	-	✔	-	-
McGready [10]	✔	✔	✔	-	✔	✔	✔	-	✔	✔	✔	✔	✔	-	-	✔	✔	-
Syhavong [17]	✔	✔	-	-	-	✔	-	-	-	✔	-	-	✔	-	-	✔	✔	-
Phuong [8]	✔	-	-	-	-	-	-	-	-	-	-	-	✔	-	-	-	-	-
Suttinont [7]	✔	-	-	-	✔	✔	-	-	-	✔	-	-	-	-	-	✔	✔	-
Ellis [11]	✔	-	-	✔	-	✔	-	-	-	✔	-	-	✔	-	-	-	✔	-
Leelarasamee [6]	✔	-	-	-	-	✔	-	-	-	✔	-	-	✔	-	-	✔	✔	-
Brown [21]	✔ [1,2]	-	-	-	-	✔	-	-	✔	✔	-	-	✔	-	-	✔	✔	-

✔=tested, -=not tested or not mentioned; Cu=culture, CBC=complete blood count, CRP=c-reactive protein, RDT=rapid diagnostic test, S=serology

[1]Flavivirus; [2]Culture

1.2 Dengue

1.2.1 The dengue virus

Dengue is caused by a small single stranded RNA (ssRNA) virus, belonging to the genus *Flavivirus*, in the Family *Flaviviridae*. The genome of the dengue virus has a single copy of positive-sense ssRNA of approximately 11 kilobases (kb) in length that encodes a single large polyprotein that is subsequently cleaved into ten structural and non-structural proteins [NS]: three structural proteins (nucleocapsid or core protein [C], precursor of membrane [prM] (membrane [M]), and envelope [E]) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The order of proteins encoded in the long, single, open reading frame is 5'-C-prM(M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3'. The virion is spherical, 40-50 nanometres (nm) in diameter, with a lipopolysaccharide envelope. The E structural protein plays a key role in several important functions of dengue viruses including receptor binding, haemagglutination of erythrocytes, and induction of neutralising antibodies and protective immune response. The E protein has the sites for viral attachment to, and transport through, host cell plasma membranes, and contains epitopes specific for serotype, dengue complex and group. Core protein is responsible for encapsidation of viral RNA genome into nucleocapsid that forms the core of a mature virion. M protein is important in the formation and maturation of the virion. NS1 plays a significant role in viral RNA replication. In addition to the E protein, NS1 is the only other viral protein that appears to play a role in protective immunity. NS3 is associated with a role in protease activity and a nucleotide triphosphatase (NTPase) or helicase activity, while NS5 has a role as the viral RNA polymerase in viral RNA replication [24-26].

There are four genetically closely related dengue virus serotypes (DENV) referred to DENV-1, DENV-2, DENV-3 and DENV-4. These are immunologically distinct,

however there is antigenic cross-reactivity of DENV1-4 antigens as each serotype shares approximately 65% of the genome, which is also similar in other flaviviruses including JE and West Nile virus [24]. Genetic variation within each serotype has been described, resulting in distinct clusters called genotypes (or subtypes) [27]. Genotypes have been identified in numerous phylogenetic studies of dengue virus serotypes based on partial or complete sequencing of different genes (e.g. E genes), gene junctions (C, prM/E, NS3) or complete genomes. The most recent review in 2011, by Chen and Vasilakis, identified genotypes of DENV1-4 based on complete E gene sequences. The analysis confirmed previously identified genotypes and also provided greater resolution for presence of new genotypes. The result has shown that DENV-1 and DENV-3 have five genotypes, DENV-2 has six genotypes and DENV-4 has four genotypes [28].

In October 2013, discovery of the new dengue virus serotype, DENV-5 was announced. The virus was isolated from a 37-year-old farmer admitted in a hospital in Sarawak state of Malaysia in 2007. It was initially thought to be the sylvatic DENV-4 which circulates among non-human primates and *Aedes nivalis* mosquitoes in the forest of SE Asia, however after full sequencing it was observed that the virus was phylogenetically distinct from the previous sylvatic DENV-4 and had some similarity with DENV-2. This was the only one case admitted to the hospital during the outbreak in Sarawak in 2007, no other cases of DENV-5 have been reported so far [29].

1.2.2 Epidemiology of dengue

1.2.2.1 Transmission

All four dengue virus serotypes are sustained in two transmission cycles: sylvatic and human cycles. The sylvatic cycle takes place in sylvan environments mainly in SE Asia and West Africa. The cycle is transmitted among arboreal canopy-dwelling *Aedes* spp. and non-human primates that appear to be the only amplification and reservoir hosts.

Ae. furcifer is one of the principal vectors in Africa and is primarily a canopy-dwelling enzootic mosquito, however they are known to move to the ground for feeding on humans. Similarly, in Asia the *Ae.* (Finlaya) *niveus s.l.* complex are a group of principal vectors that are known to feed on humans. This feeding behaviour can facilitate the movement of sylvatic strains of dengue virus from the forest to the peri-domestic environment [28]. The vectors in rural areas of Africa and SE Asia are often at high densities and dengue virus is known to transfer between non-human primates and humans. Almost all human infections are caused by dengue virus strains that circulate in domestic and peri-domestic environments where humans are the only amplification and reservoir hosts.

Different species of *Aedes* mosquitoes are responsible for transmitting dengue virus infections from person to person. The female *Ae. aegypti* remains the most efficient of the mosquito vectors mainly because of its domestic nature and the fact that it bites humans during the day. It frequently bites several times and feeds on multiple hosts during a single gonotrophic cycle [30]. Transmission to humans could occur immediately if the mosquito is disturbed during feeding from an infected host and then changes hosts or after an 8-10 day incubation period during which the virus multiplies in the salivary glands of the mosquito. Mosquitoes remain infective for life, which is usually around 30-45 days. Transovarial transmission (when the female passes the virus to her offspring) has also been reported, but the epidemiological importance of this has not been established [24, 31].

Ae. albopictus, *Ae. polynesiensis* and several species of *Ae.scutelaris* complex are also all able to transmit the dengue virus. The global distribution of only the two main vectors, *Ae. aegypti* and *Ae. albopictus,* puts nearly a third of the global human population at risk of infection [28, 31].

1.2.2.2 Global distribution and incidence

Dengue is the most important arbovirus infection in humans with a large global burden and is endemic between 30 degrees north latitude (°N) and 40 degrees south latitude (°S) (which includes all tropical and subtropical zones) wherever the environmental conditions permit dengue virus transmission by *Aedes* mosquitoes (Figure 1.4). This includes more than 100 countries in SE Asia, the Americas, the West Pacific, Africa and the East Mediterranean regions [32, 33]. There are an estimated 50-100 million dengue infections each year, resulting in 500,000 severe dengue cases and around 20,000 deaths, and approximately 2.5 billion people at risk living in dengue endemic countries. Its incidence has increased 30-fold in the past five decades [33-36]. Almost 95% of cases are children aged less than 15 years old, although there is an increase in the average age of patients with dengue infection [31, 37]. Unfortunately limited laboratory confirmation of dengue cases, low levels of reporting and inadequate surveillance worldwide means that the true distribution and public health burden are poorly described [36]. However, one of the most recent estimates of global dengue risk was created from a formal modelling framework paired with detailed longitudinal information from dengue cohort studies and population surfaces to infer the public health burden of dengue in 2010 [38]. The model developed by Bhatt and colleagues concluded that there is an estimated 390 million dengue infections per year, of which 96 million present as symptomatic infections, which is three fold higher than the figure estimated by the World Health Organization (WHO) [33].

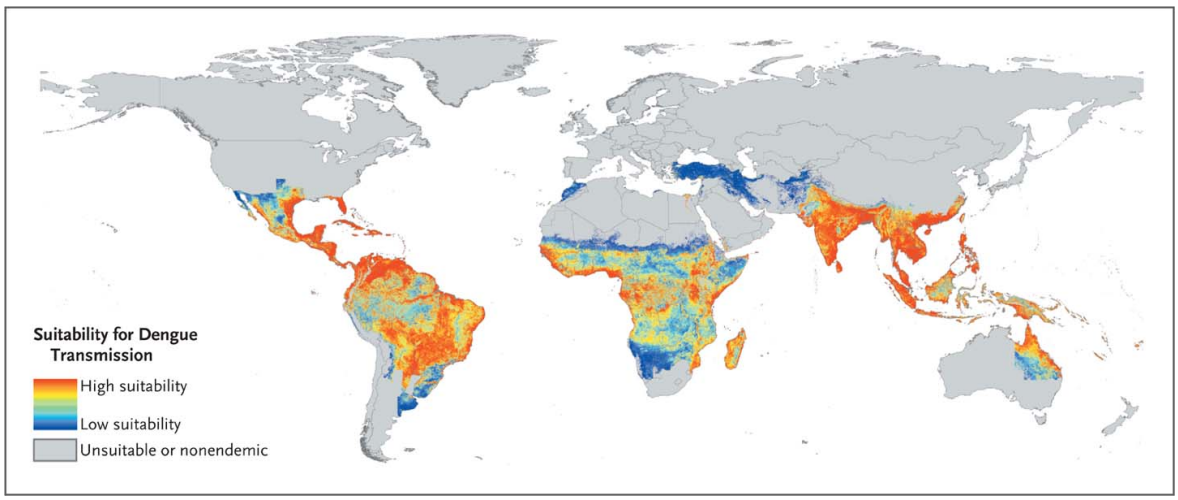

Figure 1.4 Global Dengue Risk

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Nearly three quarters of the global population who are exposed to dengue live in the Asia Pacific region. Of these, 1.3 billion people are at risk living in ten endemic countries in SE Asia [36] and dengue is a leading cause of hospitalisation and death among children in the region [39]. SE Asia has regular dengue epidemics on 3–5 year cycles [40]. Vietnam, Thailand and the Philippines reported the highest number of dengue cases among Asian countries. The Philippines reported the first dengue haemorrhagic fever (DHF) outbreak in 1953/1954, followed by a second outbreak in 1956. Thailand reported an outbreak in Bangkok in 1958. Since then the epidemic cycles in SE Asia have continued and become larger in magnitude [41, 42]. The incidence and spread of dengue fever have been increasingly reported in nine countries within the SE Asia region between 1985-2005 and have continued to increase in recent years. By 2009, almost all member countries in the region reported dengue outbreaks [43]. The case fatality rates reported approximately 1% for the region, but India, Indonesia and Myanmar where the outbreaks were far from urban areas, reported 3-5% case fatality rates [33].

1.2.2.3 Dengue in Thailand

Thailand is one of the hyperendemic countries (all four virus serotypes circulating) in SE Asia with data showing that 87% of cases are secondary infections (infected for the second or more time either with the same or different dengue virus serotype) and only 13% are primary infections (infected with the dengue virus for the first time) [41, 42]. Disease incidence and deaths remained highest in young children aged ≤15 years. Dengue is seasonal and peaks during the rainy season, the number of cases peak between May and September, although it varies slightly between regions [44]. There is no clear pattern of infecting serotype with regard to severity, although DENV-2 and DENV-3 appear to be associated with more severe symptoms than the other dengue serotypes accompanied by secondary infection. It is more likely to be a suggestive trend rather than strong evidence. The disease severity varies for individual dengue virus serotypes, year of observations or studies which may be caused by other factors and in particular the sequence of different serotypes in primary and secondary infections [45-47].

The most recent data, up until 2014, comes from the Centre of Epidemiological Information, Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand. There were around 40,000-150,000 dengue cases (total dengue including dengue fever [DF], dengue haemorrhagic fever [DHF] and dengue shock syndrome [DSS]) reported each year between 2003 and 2014, with the morbidity rate ranging from 62.59 to 241.03 per 100,000 population. The number of dengue cases and morbidity rate peaked in 2010 and 2013 with the latter reporting the highest number of cases and morbidity rate (Figure 1.5). The trend in the number of deaths and mortality rate was similar to that for the number of cases and morbidity rate which varied during the period and peaked in 2010 and 2013. However, the mortality rate remained below 0.25 per 100,000 population even during the highest morbidity rate years (Figure 1.6) [48].

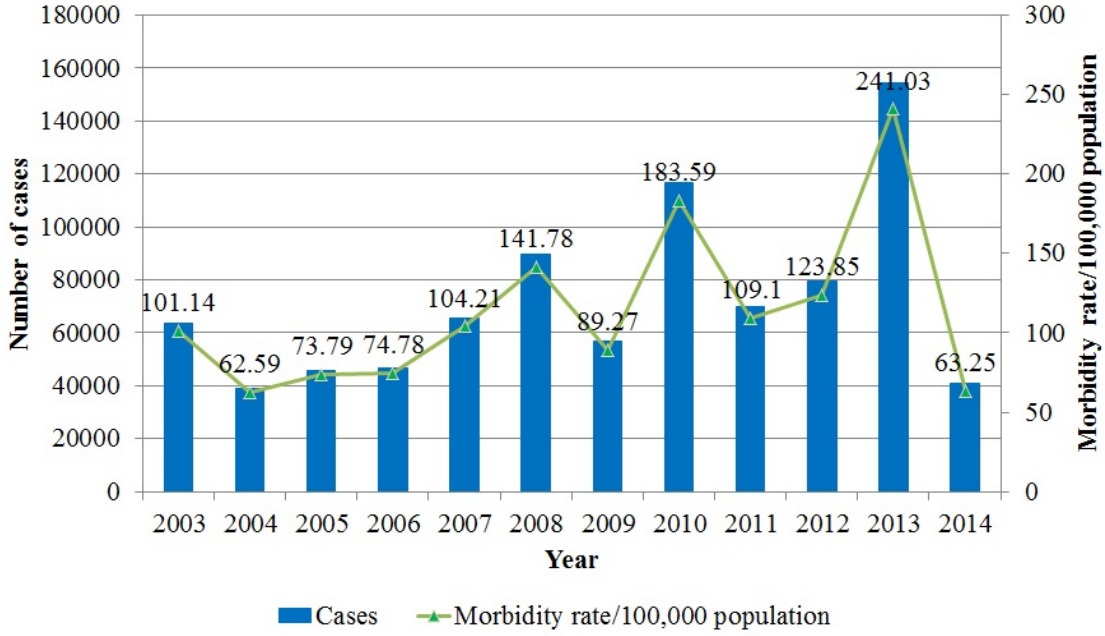

Figure 1.5 Number of cases and morbidity rate of dengue reported in Thailand between 2003 and 2014.

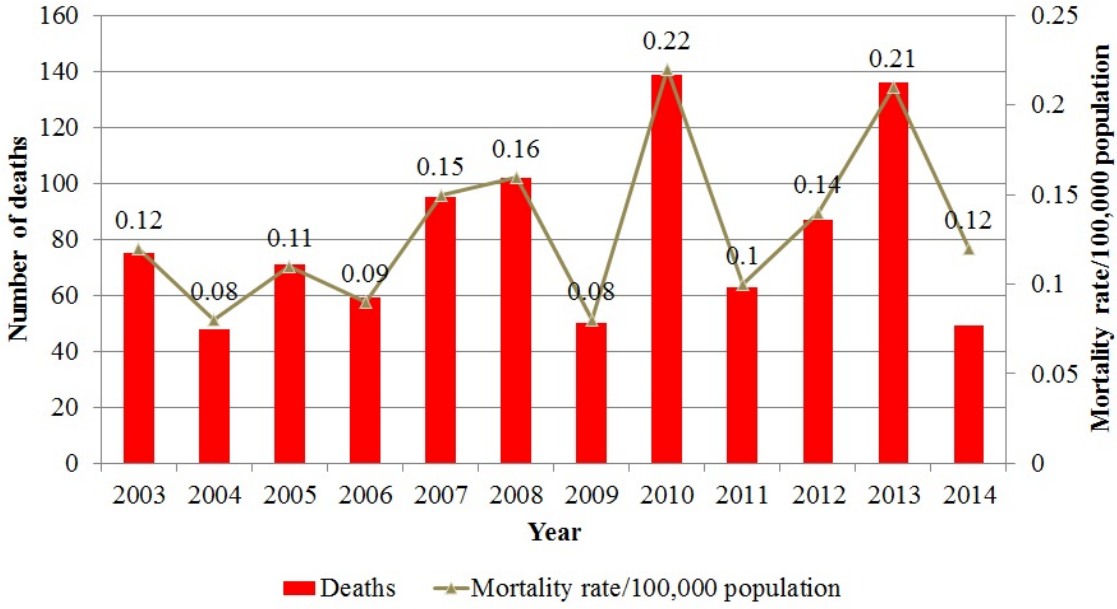

Figure 1.6 Number of deaths and mortality rate of dengue reported in Thailand between 2003 and 2014.

Note: Data from total dengue cases including DF, DHF and DSS (National Disease Surveillance, report 506 code: 66, 26, and 27, respectively).

Source for Figure 1.6 and 1.7: Centre of Epidemiological Information, Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand, http://www.boe.moph.go.th/boedb/surdata/disease.php?dcontent=old&ds=262766.

1.2.3 Clinical features

Dengue virus infection is responsible for a wide clinical spectrum of disease. Infections can cause mild acute undifferentiated febrile illness to severe disease characterised by haemorrhage and shock. All four virus serotypes (DENV1-4) share nearly identical symptoms in humans and can cause dengue fever and dengue haemorrhagic fever. Primary infection by a specific serotype induces lifelong protective immunity against the infecting serotype. A cross-protective immunity to a different serotype has been observed within 2-3 months of the primary infection, however there is no long-term cross-protective immunity [33]. Several risk factors are associated with dengue severity, including host factors, viral factors, and differences in dengue cross-reactive antibodies. The major risk factor for development of severe dengue is secondary infection with a different serotype from the first infection. Primary infection in infants born from dengue-immune mothers are at higher risk for severe infection than those born from non-immune mothers [34]. These are explained by antibody-dependent enhancement (ADE) where a dengue virus serotype uses pre-existing anti-dengue virus antibodies produced in the primary infection to facilitate entry of a different dengue virus serotype via macrophage Fc receptors [49, 50]. The immune system is tricked because the four virus serotypes have very similar surface antigens. The antibodies bind to surface antigen but do not inactivate the virus, resulting in an increased number of infected cells and a rise in viraemia. The host produces and releases cytokines and other mediators that cause an increase in vascular permeability in endothelial cells, resulting in plasma leakage. Young children are at higher risk of severe dengue as they may be less able to compensate for vascular leakage than adults and are subsequently at higher risk of dengue shock [33, 51].

In SE Asia, patients with dengue virus infection usually present with an acute undifferentiated febrile illness. The symptoms and signs are non-specific, and it is rarely possible to differentiate from antibiotic-treatable causes of fever, such as leptospirosis, typhoid and rickettsial infections [6-8, 52]. According to the WHO guideline 1997, dengue virus infections were classified into three categories: undifferentiated fever, DF and DHF, and DHF was further classified into four severity grades. The presence of thrombocytopenia with concurrent haemoconcentration differentiates grades I and II DHF from DF. DHF grade III and IV are defined as DSS [53]. Cases of DHF fulfil all of the following four clinical criteria: (1) fever or history of acute fever lasting 2-7 days; (2) haemorrhagic tendencies evidenced by at least one of the following: a positive tourniquet test; petechiae, ecchymoses or purpura; bleeding from the mucosa, gastrointestinal tract, injection sites or other locations; haematemesis or melaena; (3) thrombocytopenia ($\leq$100,000 platelets/μl); and (4) evidence of plasma leakage due to increased capillary permeability by either haemoconcentration (increase in haematocrit (Hct) $\geq$20% from average for the same age, sex and population, or decrease by $\geq$20% of baseline after intravenous fluid treatment), pleural or abdominal effusion or hypoproteinaemia. The only haemorrhagic manifestation in grade I DHF is a positive tourniquet test. In grade II, there is spontaneous bleeding, usually in the skin and other sites. Rapid, weak pulse and narrow pulse pressure of <20 (millimetre of mercury) mmHg, hypotension for age, cold, clammy skin and restlessness is considered grade III. Profound shock with undetectable blood pressure or pulse is considered grade IV. This classification continues to be widely used, despite the fact that there have been increasing concerns regarding the difficulties and complexity to use this classification system [53, 54]. The recent revision of WHO guideline 2009 classified patients as having either dengue (with/without warning signs) or severe dengue [32, 33]. Briefly, patients who live in or travel to dengue endemic areas, and present with a combination of fever and two of the following conditions are considered as

having dengue: nausea, rash, aches and pains, positive tourniquet test, leukopenia or any warning signs which requires strict observation and medical intervention (abdominal pain or tenderness; persistent vomiting; clinical fluid accumulation; mucosal bleed; lethargy; restlessness; liver enlargement >2 centimetres (cm); increase in Hct concurrent with rapid decrease in platelet count). Patients who have any of the following criteria are considered as having severe dengue: severe plasma leakage leading to shock or fluid accumulation with respiratory distress, or both; severe haemorrhage; and severe organ impairment (liver: AST or ALT ≥1,000 International Unit per litre (IU/L); central nervous system (CNS): impaired consciousness; heart: cardiomyopathy or other unusual manifestations).

After an incubation period of 3-7 days following an infective bite, the disease begins abruptly and follows three clinical phases: febrile phase, critical phase, and recovery phase (Figure 1.7).

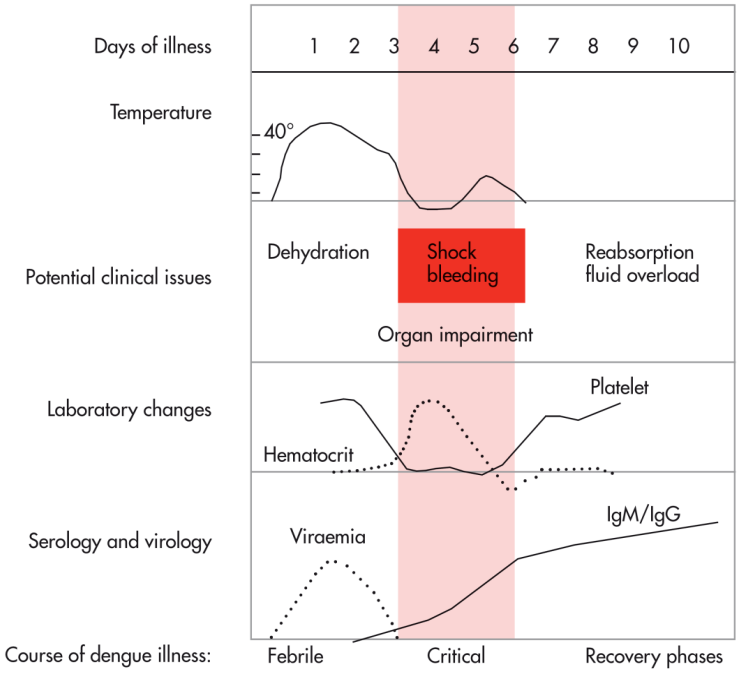

Figure 1.7 The course of dengue illness

Reproduced with permission from World Health Organization, Dengue guideline for diagnosis, treatment, prevention and control, new edition, 2009 [33].

Febrile phase

Patients typically develop a sudden onset of high fever (≥38.5°C) which is often accompanied by headache, generalised body ache, myalgia, arthralgia and vomiting, sometimes with a transient macular rash. Other symptoms, including sore throat, nausea, anorexia and vomiting, are common. Mild haemorrhagic manifestations can occur such as petechiae and bruising, particularly at venepuncture sites, and mucosal membrane bleeding (e.g. nose and gums) may be seen. A positive tourniquet test in this phase increases the possibility of dengue. The test is performed by inflating a blood pressure cuff to a midway between the systolic and diastolic pressure on the upper arm for 5 minutes and a presence of 20 or more petechiae per 2.5 square centimetres (cm^2) are considered positive. The liver can be enlarged and tender after a few days of fever. This acute febrile phase usually lasts 2-7 days and the fever can be as high as 40°C, after which most patients recover without complications. Laboratory findings include mild to moderate thrombocytopenia, leukopenia often with a moderate increase of hepatic aminotransferase levels. The white blood cell (WBC) count reaches its lowest point shortly before the temperature and platelet count drops. This marks the end of the febrile phase and beginning of the critical phase. Predicting which patients will progress to develop severe dengue is not possible and patients should be observed for warning signs [32, 33].

Critical phase

The beginning of the critical phase is around the time of defervescence, when the temperature drops to ≤37.5-38°C and remains below this level, usually on days 3-7 of illness. An increase in capillary permeability may occur at the same time with increasing haematocrit levels. The critical phase usually lasts 24-48 hours. It is crucial during the transition from the febrile phase to the critical phase for clinicians to be aware of warning signs for onset of vascular leakage. These signs include rapid decrease in platelet count and

a high or increase in Hct level, persistent vomiting, pleural effusion and ascites, increase in severe abdominal pain, tender hepatomegaly, lethargy or restlessness, and haemorrhagic manifestations. Shock occurs when a critical volume of plasma is lost due to leakage. Prolonged shock is associated with hypotension and major bleeding results in organ impairment and disseminated intravascular coagulation. In some instances, unusual manifestations including severe organ impairment such as liver failure, myocarditis, encephalopathy and/or severe bleeding may present without plasma leakage or shock.

Patients who improve after defervescence are considered to have non-severe dengue. Those who progress to the critical phase of plasma leakage without defervescence will develop warning signs and are categorised as dengue with warning signs. This group will usually recover with early intravenous rehydration, however, some cases be more serious and progress to develop severe dengue. Proper fluid resuscitation is required to prevent severe shock in patients with warning signs. If untreated, mortality can be as high as 20%, whereas appropriate case management and intravenous rehydration can reduce mortality to less than 1% [32, 33].

Recovery phase

Dengue-related vascular permeability is short-lived. After 24-48 hours, the critical phase is followed by the recovery phase where extravascular compartment fluid is gradually reabsorbed reverting to a normal level after approximately 48-72 hours. Continuing intravenous fluid or excessive fluid therapy at this stage results in a significant risk of fluid overload. Patients' symptoms are generally improved after the critical phase. Some patients may have a rash described as "isles of white in the sea of red", that appears during recovery phase. Adults may have profound fatigue or even depression for several weeks after recovery. The Hct stabilises or decreases due to the dilutional effect of

reabsorbed fluid. The WBC count usually starts to increase soon after defervescence whereas platelet count recovery typically occurs later [32, 33].

1.2.4 Laboratory diagnosis

Differentiating dengue virus infection from other infections using simple clinical and laboratory criteria in the disease endemic areas can be helpful where further tests are not available. The most common features of dengue infection are associated with haemorrhagic manifestations, particularly gum bleeding, low platelet and white blood cell count [55, 56]. However, these are not specific, and accurate clinical diagnosis is difficult due to a wide range of clinical presentations. Laboratory confirmation of dengue virus infection is therefore required for clinical management, surveillance and research to document the burden of this disease so that health planning and prevention can be applied. Rapid diagnostics are required for management of the acutely unwell patients.

Diagnostic methodologies include virus isolation, detection of viral nucleic acid, antigens and antibodies specific to the dengue virus or a combination of these tests. The timing of specimen collection and infection status, whether the infection is primary or secondary are critical for test choice (Figure 1.8).

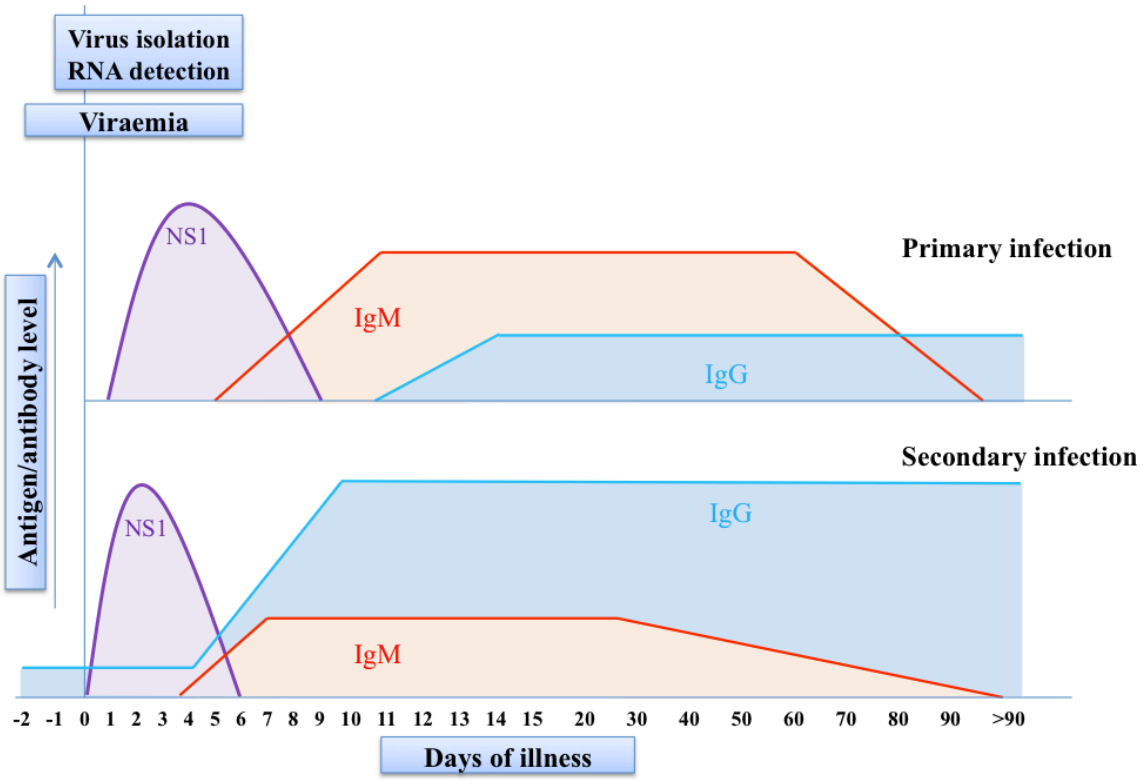

Figure 1.8 Detection opportunities for laboratory diagnostic methodologies of dengue.

NS1 denotes non-structural protein 1

Adapted with permission from World Health Organization, Dengue guideline for diagnosis, treatment, prevention and control, new edition, 2009 [33].

During the acute phase of disease, dengue virus and its components (RNA or antigen (NS1)) can be detected in whole blood, plasma, serum, and infected tissues from 0-7 days after onset of fever and correlate with fever duration. Virus isolation, nucleic acid or antigen detection methods are not routinely performed by clinical laboratories. Serological tests such as Enzyme-linked immunosorbent assay (ELISA) are more commonly used and seroconversion of immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies is the standard for serological confirmation of dengue infection [33]. In primary infection, IgM antibody is detected typically five or more days after onset of fever. IgM level peaks

about two weeks after onset of symptoms and lasts for 2-3 months, whereas IgG antibody is detected later with low titre from 8-10 days after onset of fever. It can be detectable for several months or probably even for life. In secondary infection, IgM antibody appears earlier or in the same time frame as primary infection but titres are usually lower than those obtained in primary infection and can be undetectable in some cases. IgG antibody appears rapidly with high titres soon after onset of fever and persists for 10 months to life [33, 34, 57]. Highly accurate laboratory diagnostic tests usually require more complicated techniques and skilled personnel while rapid point-of-care diagnostic tests have lower accuracy. Virus isolation and nucleic acid detection are more specific than serological tests, but are also expensive and labour-intensive. Figure 1.9 shows a comparison between the accessibility of the diagnostic tests and the confidence in the results of the test.

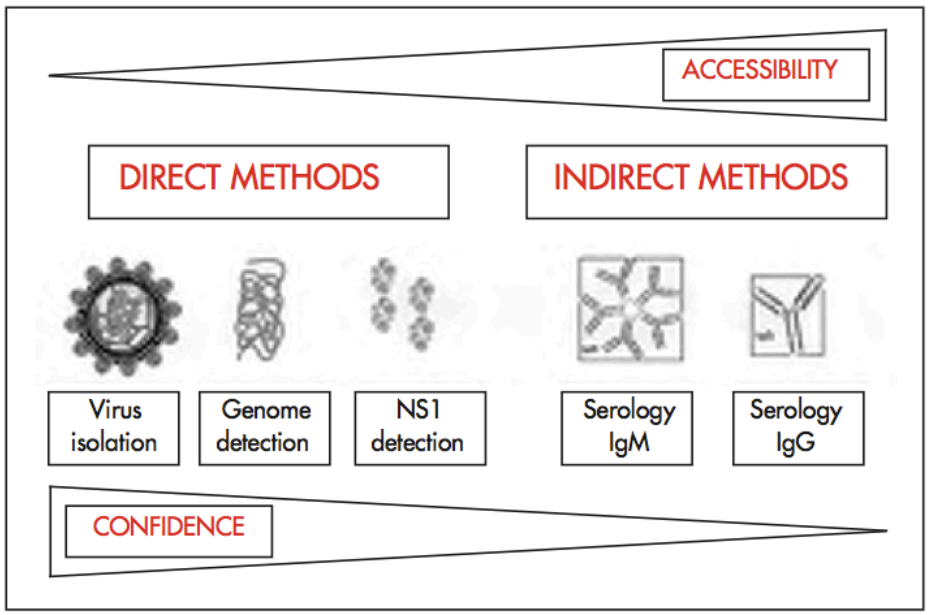

Figure 1.9 Comparison of diagnostic tests according to their accessibility and confidence.

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1.2.4.1 Isolation of dengue virus

Virus culture is the definitive diagnostic test, since it is highly specific, and remains the gold standard for detection of dengue virus infection, in spite of the fact that it usually requires several days to weeks to complete. There have been four methods of viral isolation used for dengue viruses: inoculation of diagnostic specimens intrathoracically into adult mosquitoes (*Ae. aegypti, Ae. albopictus*, and *Toxorhynchites splendens*), intracerebrally into newborn mice, into mammalian cell cultures (using Vero, LLCMK2, and BHK21 cell lines), and into mosquito cell cultures (using C6/36 or AP61 cell lines from *Ae. albopictus or Aedes pseudoscutellaris,* respectively) [58].

Mosquito inoculation is the most sensitive method, but the least used, as it needs insectaries to produce large numbers of mosquitoes for inoculation and isolation precautions to avoid the release of infected mosquitoes. Mosquito cell culture is the most widely used method of dengue virus isolation for routine diagnosis where laboratory facility is available. Mammalian cell cultures may be used but are less efficient. Mouse brain inoculation is expensive, slow, and has low sensitivity therefore it is not routinely used and is no longer recommended for viral isolation. Optimal specimens for virus isolation should be collected during viraemia, usually before five days of illness. Specimens can be whole blood, plasma, serum or tissues collected from fatal cases (i.e. liver, lung, spleen, lymph nodes, thymus, bone marrow and other tissues). Prior to culture, specimens should be kept at 4-8°C for up to 24 hours or kept frozen at -70°C or liquid nitrogen for long-term storage [33, 57, 58].

Jarman *et al.,* has reported that the virus isolation rate was 62.5% when using direct C6/36 cell culture. Specimens that were negative for direct C6/36 isolation were inoculated into *T. splendens* followed by C6/36 cell culture and this increased the isolation rate up to 79.4%. The virus isolation positivity rate of the four serotypes ranged between 71.5-84.2%. The rate was higher in patients with primary infection than those with secondary infection

(91.0 and 77.6%, respectively). Moreover, specimens collected early in the course of illness had a higher positivity rate: 85.3% when collected before or at day four of illness and 65.4% when collected later than day four. This study suggested that the factors that might influence overall performance of virus isolation included higher viraemia, lower level of anti-dengue IgM and IgG, days of fever (≤4 days), primary infection, high body temperature (≥38°C) with a final clinical diagnosis of dengue, and infecting serotype (DENV-4) [59].

Other advantages beside diagnosis are that virus isolates can be kept and characterised for further studies such as genome sequencing, virus-antibody interactions, and infectivity measures. Isolation followed by an immunofluorescence assay (IFA) for confirmation generally takes 1-2 weeks and is possible only if the specimen is properly stored [33]. Laboratory equipment is expensive and culture requires experienced technicians to develop and maintain cell cultures. Hence, this method is not widely available in most developing countries and not widely used in routine diagnostic laboratories.

1.2.4.2 Antigen detection

NS1 is a highly conserved glycoprotein produced by all flaviviruses and is the major diagnostic marker for dengue virus infection. NS1 is produced from dengue-virus infected cells as soluble monomers that dimerised in the lumen of endoplasmic reticulum and is subsequently transported to the cell surface where the protein remains membrane-associated or is secreted into extracellular milieu as a soluble hexamer [60]. NS1 can be detectable in the bloodstream from the first day after onset of illness up to day nine, when the clinical phase of disease is over. Alcon *et al*. reported that NS1 levels in primary infection ranged from 0.04 to 2 micrograms per millilitre (μg/ml) in acute phase serum specimens (day 0-7) and 0.04μg/ml in a convalescent phase specimen (day 8 and later). In secondary infection, levels ranged from 0.01 to 2μg/ml in acute phase serum specimens

and NS1 was not detectable in convalescent phase serum specimens [61]. A study, limited to DENV-1, reported that NS1 can persist until 18 days after onset of illness with a peak at days 6-10, however this study was limited to the small number of specimens tested after 10 days of fever and only one specimen was positive on day 18. The authors did not indicate the status of infection whether primary or secondary infection [62]. The NS1 level in plasma has been found to correlate with viraemia titre and disease severity [63].

Many studies have investigated the utility of rapid immunochromatographic tests (ICT) and ELISAs, targeting NS1 as a diagnostic tool during acute phase of infection [64-66]. The ICT format has several advantages compared to the ELISA format such as being simple to perform, and results being available within minutes. The most recent meta-analysis of NS1 was reported in 2014 [67], analysing 18 studies including ELISA and ICT formats. The pooled sensitivity and specificity of the ELISA format (analysed from 14 studies) were 67.0% (95% CI 59.0-74.0) and 99.0% (95% CI 97.0-99.0), respectively. For the ICT format, the pooled sensitivity and specificity (analysed from nine studies) were 71.0% (95% CI 61.0-79.0) and 99.0% (95% CI 98.0-100), respectively. NS1 tests have been applied to serotype specific identification assays. The highest accuracy for serotyping was obtained from 2/6 analysed studies, 82.4 and 80.5%, using Dengue NS1 Ag STRIP kit. The kit was shown to be the best test for confirmation and serotype identification among the tests used in the analysis [67]. The complementarity with antibodies is the most important aspect of the NS1 detectability, since the positivity of viraemia and virus RNA detections are normally concomitant with the NS1 positivity. In an attempt to improve the sensitivity of the assay, combined NS1-based capture and IgM assays have been evaluated in three studies. Using these assays the pooled sensitivity increased to 83.0% (95% CI 68.0-92.0), however the specificity decreased to 86.0% (95% CI 79.9-01.0) [67]. This is possibly due to false positive IgM results as a positive IgM on a single specimen cannot not confirm acute dengue infection.

In summary, NS1 antigen detection has shown generally very high specificity for confirmation of dengue infection, however some manufacturers' tests are not as specific as they should be. The sensitivity varies depending on primary or secondary infection, days of fever, the NS1 kit/test format, and which gold standard comparators are used. Whole blood, plasma and serum are commonly used for NS1 antigen detection assays. Urine specimens may be useful when serum cannot be collected and in limited resource settings, however the positivity rate was low, ranging from 13.0-43.0%, in one study [68]. Other specimen types, such as tissue sections, can be used to detect dengue antigens by immunohistochemistry [57].

1.2.4.3 Antibody-based diagnosis

Antibody-based serological diagnostic methods play a significant role and have been widely used for the diagnosis of dengue infection. The haemagglutination inhibition (HI) test was the standard method due to its high sensitivity, relatively ease, and because it can differentiate between primary and secondary infections. However, the lack of specificity, requirement for paired samples and the fact that it is unable to identify infecting serotype are limitations of this method [58]. The complement fixation (CF) test is not commonly used for routine diagnosis, although it is very specific in primary infection and can detect the infecting serotype. It is difficult to perform [25, 58]. The plaque reduction neutralisation test (PRNT) is used to quantify the titre of neutralizing antibodies that inhibit dengue virus infection. PRNT is not routinely used for dengue diagnosis as it is time consuming, labour intensive and low throughput, although it remains widely used for immunity studies [51] and dengue vaccinology [69].

ELISAs have been widely used and are considered to be the most useful tests due to their technical feasibility and high sensitivity. Innis and colleagues have evaluated the IgM and IgG antibody-based ELISA to replace the HI test as the gold standard serological test [70]. This study showed that the sensitivity of the IgM antibody ELISA was 78.0% (95%

CI 75.0-81.0) when evaluated using acute serum specimens and 97.0% (95% CI 96.0-98.0%) using paired serum specimens as compared to the HI test. Primary and secondary infection could be classified using the ratio of anti-dengue IgM to IgG antibody ELISA unit. The IgM and IgG antibody ELISA has been widely used as gold standard serology for dengue infection instead of the HI test [71]. There are commercially available test kits for semi-quantitative detection of IgM and IgG antibodies [72, 73]. The sensitivity and specificity of the IgM antibody ELISA ranged from 61.5-99.0% and 79.9-100%, respectively.

Most of the dengue serodiagnostic assays require paired specimens to confirm infection, which results in delayed diagnosis of acute dengue infection and are not usually helpful for patient management. Attempts to find sensitive and specific tests for confirmation of infection from single acute specimens in patients with acute dengue infection have focused on evaluation and comparison of ELISA test kits as well as rapid diagnostic tests which potentially could be used in the field [71, 73-75]. The use of salivary (Immunoglobulin A) IgA antibody ELISA for early dengue detection has been reported recently. This may be useful in dengue endemic areas where the majority of cases are secondary infection when sensitivity was 100% in the first three days of fever with 97.0% specificity. However, the sensitivity was low in primary infection (36.0%) [76].

The IgM antibody-based ICTs have been evaluated for use as rapid diagnostic tests for dengue virus infection [71]. Early RDTs for dengue IgM antibody detection had low sensitivity, ranging from 6.4-65.3%, with specificities ranging from 69.1-100% in one comparison [71]. Newer RDTs, such as the Standard Diagnostics (SD) IgM, have been demonstrated to have improved sensitivity (79.2%) and specificity (89.4%) [77]. Addition of NS1 antigen detection has been shown to improve the sensitivity (92.9%) [77].

The addition of NS1 antigen detection to the combination of antibody-based detection ICT improved the detection accuracy of the test and extends the possible window to detect early acute specimens [77-79].

The limitation of these antibody-based techniques is cross-reactivity between all four serotypes of dengue virus and other flaviviruses [58] and therefore testing on paired specimens is still considered the most reliable serodiagnostic test for dengue [56].

1.2.4.4 Molecular-based diagnosis

Many molecular techniques for dengue viral RNA detection have been published. Reverse transcriptase polymerase chain reaction (RT-PCR), nested-RT PCR and real-time RT-PCR (rRT-PCR) have been developed for rapid detection and typing of dengue infection (group and serotype specific) from clinical specimens [80-86]. Among these PCR assays, a nested-RT PCR developed by Lanciotti *et al.* [80], single tube multiplex RT-PCR by Harris *et al.* [81], and one-step SYBR Green I-based rRT-PCR by Shu *et al.* [86] are well known and well validated. PCR was found to be useful in the early phase of dengue infection, being more sensitive than virus isolation, able to confirm the diagnosis and able to identify the infecting serotype from clinical specimens [87]. A prospective study evaluating diagnostic tests for detection of acute dengue infection on single acute specimens demonstrated the usefulness of rRT-PCR for rapid and early detection, with high sensitivity and specificity (88.9 and 95.6%, respectively) [16].

Recently, several isothermal-based molecular techniques have been developed to overcome the limitations of PCRs. These techniques do not require an expensive thermocycler, just a water bath or heat block is sufficient. They are quick and easy to perform compared to RT-PCR. One of the techniques, transcription-mediated amplification (TMA), was shown to be highly sensitive: it could detect dengue RNA in approximately 89.0% of acute phase clinical specimens. This technique was 10 to 100 times more sensitive as compared to rRT-PCR [88]. Another isothermal-based molecular tool, real-

time reverse transcription-loop-mediated isothermal amplification (RT-LAMP), has been developed for rapid detection and differentiation of dengue virus serotype. RT-LAMP reactions are performed at 60°C for more than 30 minutes. The results are interpreted by either visualised turbidity or quantitative fluorescence detection using SYBR Green. Several studies reported the usefulness of this technique as simple and potentially highly feasible in clinical settings [89, 90]. The most recent technique, recombinase polymerase amplification assay (RPA) has been developed for rapid diagnosis of dengue infection and is proposed to be simpler than the RT-LAMP. This technique is performed at 39-42°C for less than 30 minutes and is performed using a portable fluorescence-reading device [91]. Only one pair of primer is required, rather than three pairs for RT-LAMP [90], in which multiplexing the target is possible. A study evaluating this assay in two field sites: Kedougou (Senegal) and Bangkok (Thailand), showed high diagnostic accuracy of the test. The sensitivity and specificity were 98.0 and 100% for Senegal, and 72.0 and 100% for Thailand, respectively [91].

A study by De Paula *et al.*, that investigated different specimen types for dengue viral detection by RT-PCR, showed that serum is the best clinical sample for RT-PCR amplification [92]. In contrast, in a study from Thailand that compared the results of RT-PCR with the use of whole blood and plasma or serum revealed that RNA extracted from whole blood followed by RT-PCR resulted in higher detection rate than the use of plasma or serum (74.0 and 52.0%, respectively). Moreover, RT-PCR was able to detect viral RNA up until day nine of illness when using whole blood, whereas the use of plasma/serum allowed the detection of viral RNA until day seven [93].

1.3 Leptospirosis

1.3.1 The Leptospira

Leptospires are thin spirochaetes, corkscrew-shaped bacteria, which differ from other spirochaetes by the presence of end hooks (Figure 1.10). The organism is approximately 0.25 x 6-25 micrometres (μm) in size, hooked at one or both ends, highly motile, and is an obligate aerobe. They are catalase and oxidase positive. Spirochetes share features of both Gram-positive and Gram-negative bacteria [94]. Similar to other spirochaetes, leptospires have a typical double membrane structure, in which the cytoplasmic membrane is closely associated with the peptidoglycan cell wall as in Gram-positive bacteria. They also have an outer membrane in which the lipopolysaccharide (LPS) composition is similar to that of Gram-negative bacteria. However, it appears to be fluid and labile and has relatively low endotoxic activity, which is different from the outer membrane of Gram-negative bacteria [95, 96]. Since the organism stains poorly and is too thin to be visible by conventional light microscopy, dark-field or phase-contrast microscopy of wet preparations is required for direct visualisation of leptospires [94].

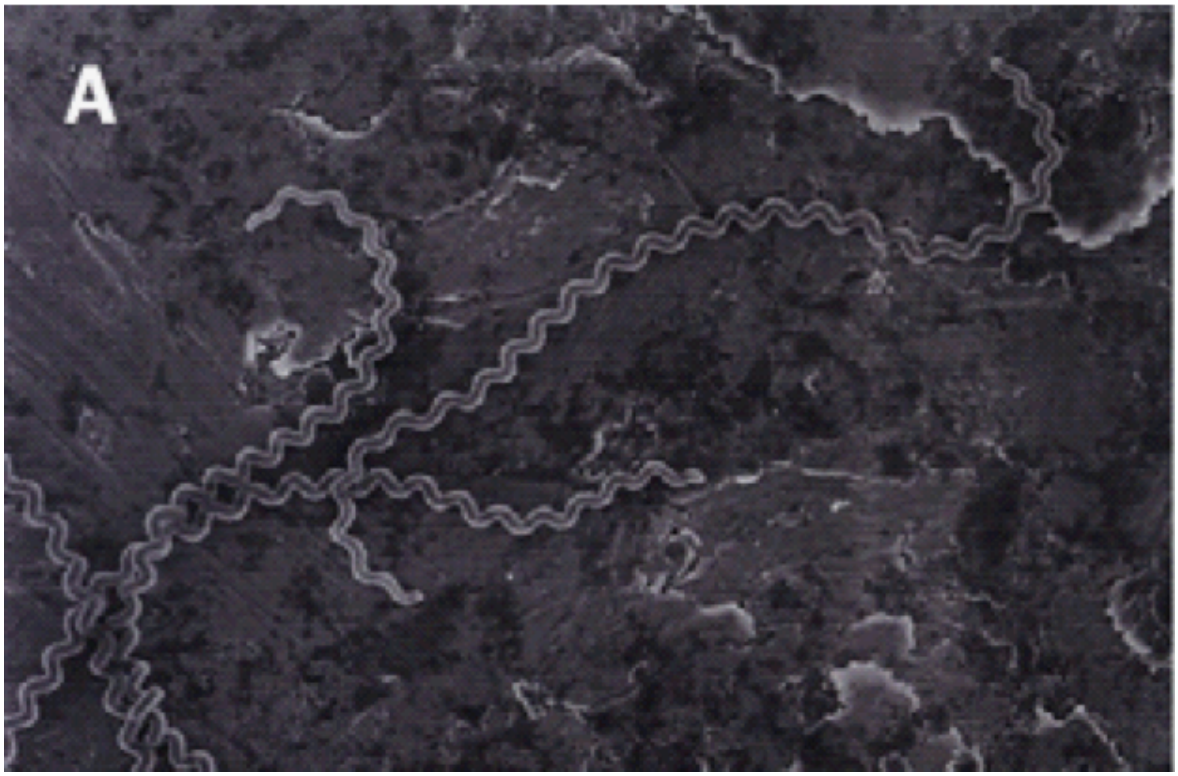


Figure 1.10 High-resolution scanning electron micrograph of *Leptospira interrogans* serovar *copenhageni* showing characteristic hooked ends.

Reprinted from Bharti, A.R., Nally, J.E., Ricaldi, J.N., Matthias, M.A., Diaz, M.M., Lovett, M.A., Levett, P.N., Gilman, R.H., Willig, M.R., Gotuzzo, E., and Vinetz, J.M., *Leptospirosis: a zoonotic disease of global importance.* Lancet Infect Dis, 2003. **3**(12): p. 757-71 with permission from Elsevier [94].

Leptospires belong to the genus *Leptospira,* family Leptospiraceae, order Spirochaetales [96]. *Leptospira* is divided into several species and subspecies, called serogroups and serovars. Serogroups comprise antigenically related serovars, usually associated with a natural host [97]. The classification of *Leptospira* has undergone many changes over time. Traditionally, the genus *Leptospira* was divided into two serological species, *Leptospira interrogans* and *Leptospira biflexa.* The former comprises all pathogenic strains and the latter contains the saprophytic strains. Both species have been divided serologically into numerous serovars using the cross-agglutination absorption test

(CAAT) [97, 98]. More than 200 serovars of *L. interrogans*, categorized into 24 serogroups, and more than 60 serovars of *L. biflexa* have been recognized [98]. The currently used genetically-based classification system indicates that at least 20 species can be identified by deoxyribonucleic acid (DNA) hybridization analysis [97]. Phylogenetic analysis of the 16S ribosomal ribonucleic acid (*rRNA*) gene sequence divides *Leptospira* species into three groups based on the pathogenicity status: pathogenic, saprophytic, and intermediate species with unknown pathogenicity [97, 99]. The current schematic classification of the organism (Figure 1.11) includes eight pathogenic, seven non-pathogenic, and five intermediate *Leptospira* species [97, 99, 100].

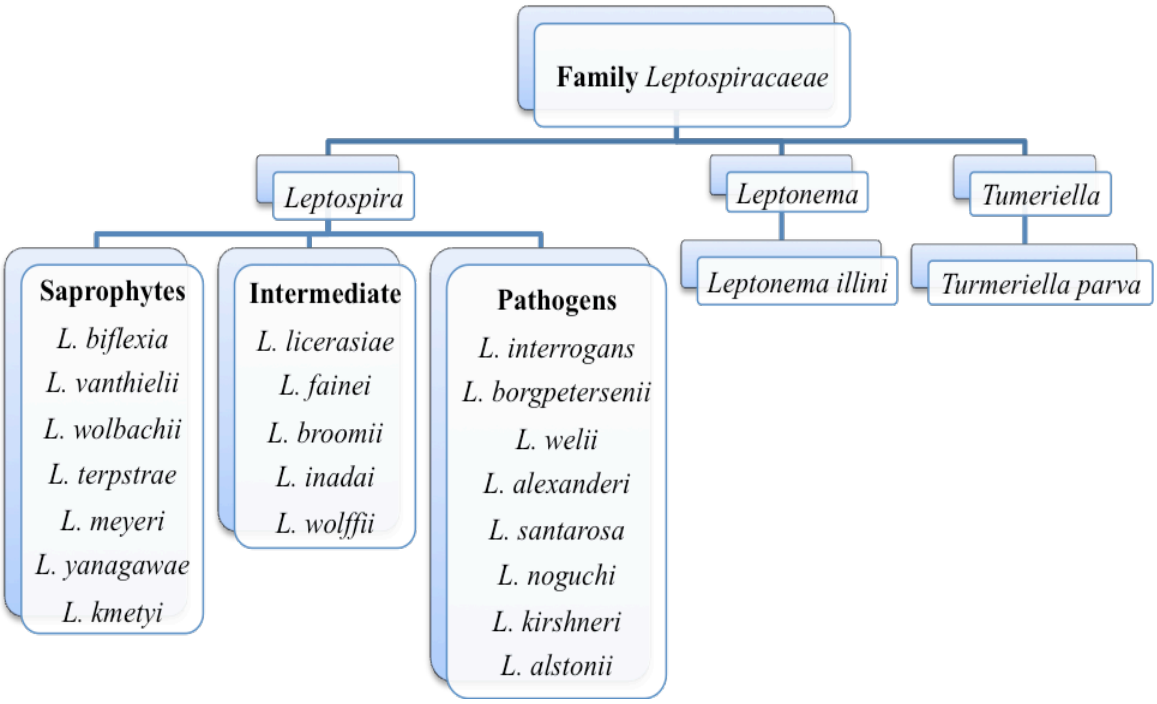


Figure 1.11 Classification of *Leptospira* [97, 99, 100].

The serological and genetic based identification and classification systems are particularly complicated and poorly correlated: for example, serovars of the same serogroup may be distributed among different species, and pathogenic and non-pathogenic serovars appear within the same species [97, 98]. Moreover, the retention of the *L. interrogans* and *L. biflexa* as specific names in the genomic classification leads to

confusion in the nomenclature of Leptospires. The recommendation currently used suggests species name followed by the term ‘ serovar’ and serovar name with initial capital letter and non-italic, for example *Leptospira interrogans* serovar Autumnalis [101].

A great deal of work in the last decade has led to publications on the whole genome sequence of two pathogenic strains (*L. interrogans and L. borgpetersenii*) and one non-pathogenic strain, the saprophyte *L. biflexa* [102-105]. The genome sizes of *Leptospira* spp. are approximately 3.9-4.6 megabases (mb) located on two circular chromosomes. A complete genome sequence of a representative virulent serovar type strain (Lai) of *L. interrogans* serogroup Icterohaemorragiae consists of a 4.33 mb large chromosome and a 359 kb small chromosome, with total of 7,768 predicted genes [102]. The much larger size of the leptospiral genome compared to other spirochaetes such as *Treponema* sp. has been suggested as one explanation for the ability of *Leptospira* to live in several different environments and hosts [102, 103].

1.3.2 Epidemiology of leptospirosis

1.3.2.1 Transmission

Leptospirosis is an infectious disease caused by pathogenic leptospires that are transmitted to humans by either direct or indirect contact with the urine of an infected animal. Many domestic animals including dogs, pigs, and rodents are important sources of human infection as they are the major natural reservoirs and carriers [98]. Humans are mostly a dead-end host and are infected by direct contact with infected soil or water. Occupational exposure is important. Farmers, fish workers, miners, veterinarians, military personnel or sewer workers who work in dirty areas and have contact with infected animals or exposure to water or soil contaminated by infected animal urine or body fluids are at high risk of infection. Similarly, people living in poor housing with lack of adequate sanitation are also at risk of exposure to leptospires in both rural and urban slum

communities. In addition, people involved in outdoor freshwater leisure activities are also at risk. Besides regional environmental and occupational exposure, international travel to disease endemic areas is also a risk factor for disease acquisition [106, 107].

Leptospires are naturally sustained by chronic infection or persistent colonisation of the renal tubules of maintenance hosts after primary infection. The hosts or carriers can remain asymptomatic and can excrete leptospires in their urine for long periods of time or even for their entire lives. Organisms are usually transferred from animal to animal by direct contact. Leptospires survive in moist and warm soil, stagnant water at neutral or alkaline pH, favouring survival in tropical environments [94]. Human to human transmission occurs only very rarely and has never been proven to be an important source of transmission [94, 108].

1.3.2.2 Global distribution and incidence

Leptospirosis is one of the most widespread zoonoses in the world, present in both tropical and temperate zones. The incidence of human infection is significantly higher in the tropics than in the temperate regions mainly because leptospires can survive longer in warm and humid conditions. The disease occurs in both industrialised and developing countries, however endemicity in low-income countries is traditionally strongly associated with local environment conditions and occupational exposure as humans are accidentally infected through contact with contaminated water [94, 98]. More recently, leptospirosis has been highlighted as an emerging zoonotic bacterial infection in part because of infection related mortality following natural disasters in dense urban populations as reported after Typhoon Ondoy in the Philippines in 2009 [109]. It is of major importance not only in human public health but also in animal production worldwide.

The true global incidence of leptospirosis is largely unknown, and is likely to be underestimated for a number of reasons, including the lack of awareness amongst

healthcare workers, meaning the disease is under-recognised, as well as inadequate diagnostic tools and the difficulties of confirming the diagnosis [110]. In addition, to complicate matters, many infections in endemic settings are asymptomatic or subclinical [94] and there is a lack of notification systems, or notification is not mandatory, in the vast majority of countries [107]. To address these problems the WHO set up a group known as the Leptospirosis Burden Epidemiology Reference Group (LERG), which aims to assess the global burden of human leptospirosis. The second meeting of LERG from 2010 generated a systematic literature review that estimated the median global annual incidence of endemic (excluding outbreak cases) and epidemic human leptospirosis as five and 14 per 100,000 population, respectively [111]. The incidence of endemic human leptospirosis varied by region, with median (range) incidences per 100,000 population from highest to lowest of: Africa 95.5 (62.8-160.2), West Pacific 66.4 (1.1-975.0), America 12.5 (0.1-306.2), SE Asia 4.8 (0.3-7.3) and Europe 0.5 (0.1-15.8). This is in reasonable agreement with previous incidence estimates from 2003. Disease incidence of more than 100 per 100,000 may occur during outbreaks and in high-exposure risk groups [108]. The geographical distribution of leptospirosis is shown in Figure 1.12 [112]. The annual incidence of human leptospirosis data derived from official data from 1996 onwards for certain countries, including the 28 countries with the highest incidence are listed in Figure 1.13 [112].

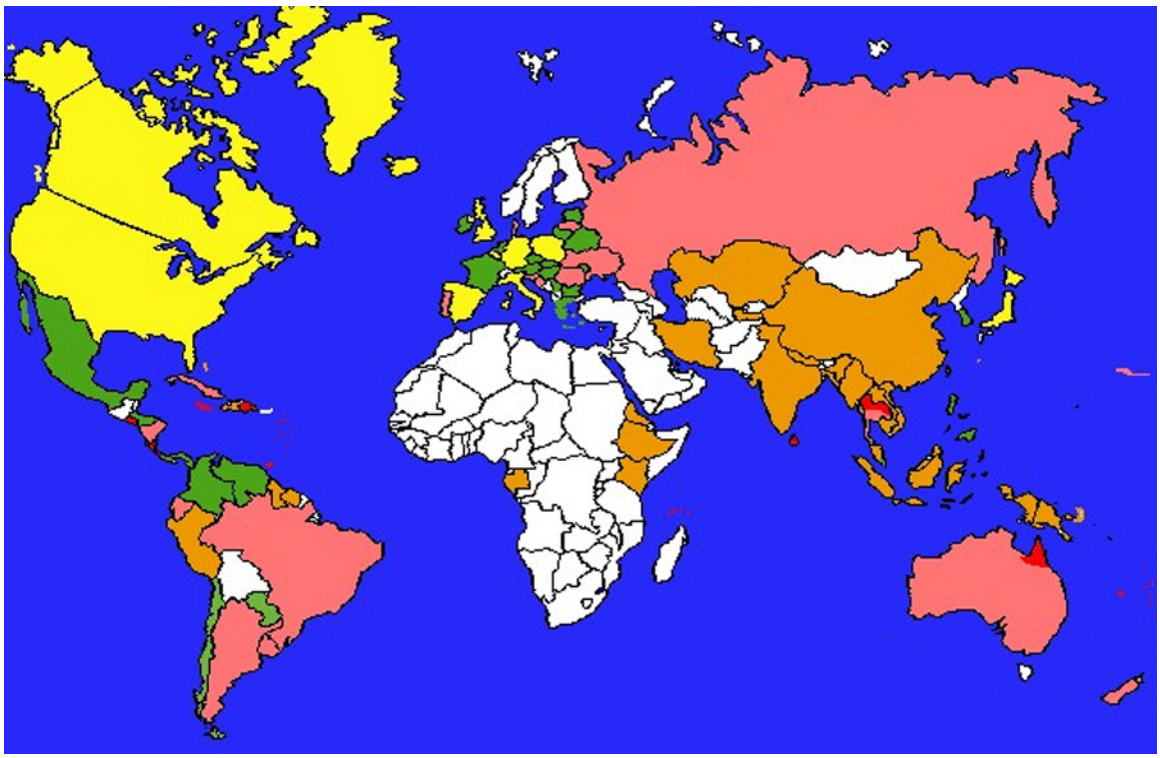


Figure 1.12 Global annual incidence of human leptospirosis.

Colours reflect incidence, in declining order: red, pink, green, yellow. Gold reflects areas with probable, but not estimated, high incidence. White reflects absence of data.

Reprinted from Pappas, G., Papadimitriou, P., Siozopoulou, V., Christou, L., and Akritidis, N., *The globalization of leptospirosis: worldwide incidence trends.* Int J Infect Dis, 2008. **12**(4): p. 351-7 with permission from Elsevier [112].

Countries with the highest incidence			Countries for which no data are available, probably endemic	Other countries	
Rank	Country	Annual incidence per million population		Country	Annual incidence per million population
1	Seychelles	432.1	India	Belarus	3.4
2	Trinidad and Tobago	120.4	Malaysia	Bulgaria	3.7
3	Barbados	100.3	Bangladesh	Chile	1.6
4	Jamaica	78	Vietnam	Colombia	1.6
5	Costa Rica	67.2	Laos	Czech Republic	1.8
6	Sri Lanka	54	Nepal	France	3.9
7	Thailand	48.9	Cambodia	Germany	0.7
8	El Salvador	35.8	Indonesia	Greece	3
9	New Zealand	26	Myanmar	Honduras	3.1
10	Uruguay	25	China	Hungary	3.1
11	Cuba	24.7	Iran	Ireland	2.2
12	Nicaragua	23.3	Suriname	Italy	0.7
13	Croatia	17.3	Haiti	Lithuania	2.2
14	Russia	17.2	Peru	Mexico	1
15	Ukraine	15.3		Netherlands	1.9
16	Dominican Republic	13.8		Panama	1.3
17	Brazil	12.8		Paraguay	1.9
18	Ecuador	11.6		Serbia and Montenegro	1.5
19	Argentina	9.5		Singapore	2
20	Romania	9.4		South Korea	2.8
21	Australia	8.9		Spain	0.3
22	Portugal	6.8		UK	0.6
23	Denmark	6		USA	0.1
24	Latvia	5.6		Venezuela	3.8
25	Slovenia	5.4			
26	Philippines	4.8			
27	Slovakia	4.4			
28	Taiwan	4.1			

Figure 1.13 Annual incidence of leptospirosis worldwide.

Reprinted from Pappas, G., Papadimitriou, P., Siozopoulou, V., Christou, L., and Akritidis, N., *The globalization of leptospirosis: worldwide incidence trends.* Int J Infect Dis, 2008. **12**(4): p. 351-7 with permission from Elsevier [112].

1.3.2.3 Leptospirosis in Thailand

In Thailand, leptospirosis is endemic with the number of cases reported ranging from 100-400 cases per year in the decades prior to 1996. The number of cases suddenly increased to over 2,000 cases per year in 1997 and reached 6,000 cases in 1999. A remarkable change occurred in the year 2000 with more than 14,000 cases [113]. This peak was followed by a gradual decline to below 3,000 cases in 2005. After that, the number of cases has stabilised at around 2,000-5,000 cases per year (Figure 1.14). The number of deaths and mortality rate per 100,000 population is shown in Figure 1.15. Most cases throughout this period were reported from Northeast Thailand. As previously highlighted

mortality data is unreliable due to the routine surveillance system used. A study of flood data from 2010-2012 examined the temporal and spatial patterns of leptospirosis, its association with flooding and animal census data in Thailand. The results showed a spatial association of leptospirosis with location in Northeast Thailand but no significant association with flooding. The high incidence of the disease in the Northeast region was explained by agriculture and animal farming activities in the region [114].

The number of leptospirosis cases reported in Thailand by the Bureau of Epidemiology, Department of Disease control, Ministry of Public Health demonstrated the seasonal variation in disease occurrence, with most cases occurring during the rainy season, July to October [113]. This is in line with the finding of a study from patients with clinically suspected leptospirosis in nine provinces, representing all regions of Thailand. The study could confirm the diagnosis in 20% of cases and most cases presented in the rainy season [115].

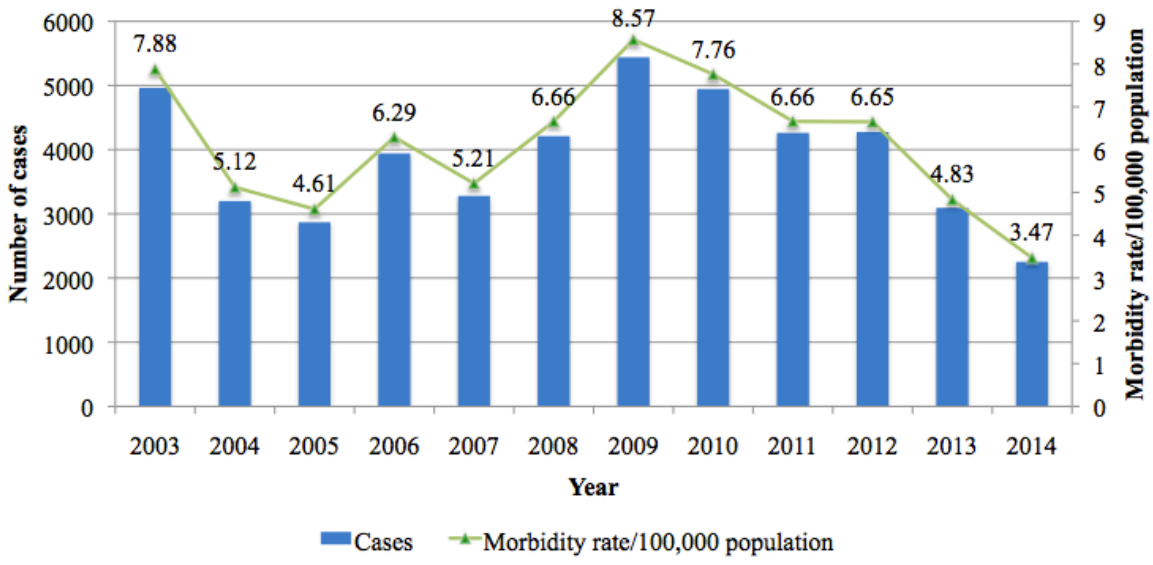


Figure 1.14 Number of cases and morbidity rate of leptospirosis reported in Thailand between 2003 and 2014.

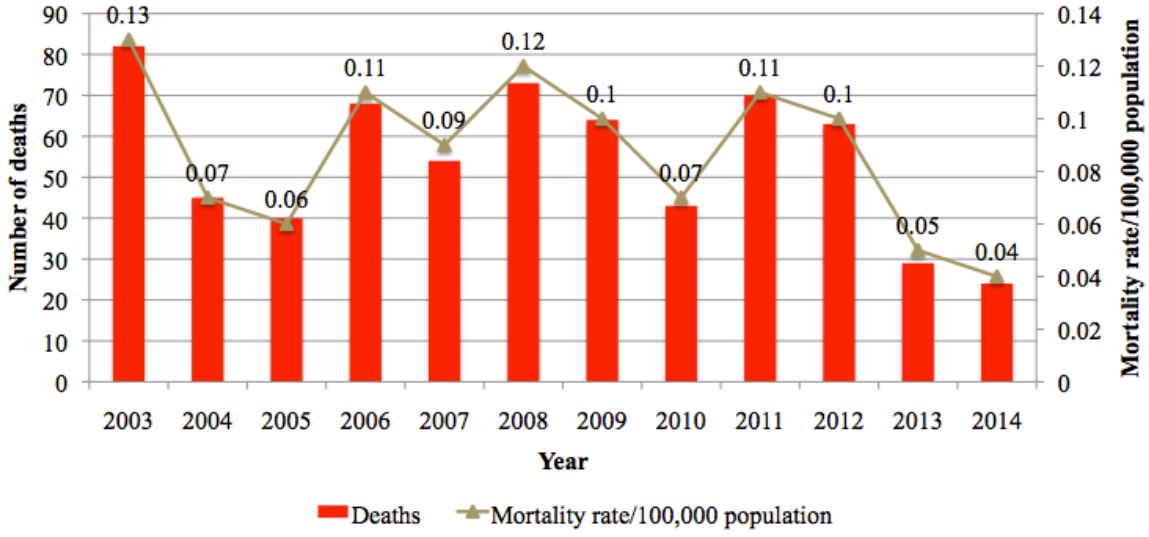


Figure 1.15 Number of deaths and mortality rate of leptospirosis reported in Thailand between 2003 and 2014.

Source for Figure 1.14 and Figure 1.15: Centre of Epidemiological Information, Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand, http://www.boe.moph.go.th/boedb/surdata/disease.php?dcontent=old&ds=43.

1.3.3 Clinical features

The clinical features of leptospirosis are highly variable ranging from a mild, influenza-like illness or self-limited febrile illness to a life threatening illness. While the illness develops, a broad spectrum of organ systems may be involved, reflecting the systemic nature of infection. Clinical diagnosis is difficult due to the variation and non-specific clinical manifestations. It is often confused with other diseases such as influenza, scrub typhus and dengue fever [107, 108].

Leptospira enters the human body via cuts or abrasions of the skin through mucous membranes with an incubation period of 5-14 days (range of 2-30 days) [108]. In 1967, Turner described the clinical presentation of leptospiral infection as having two overlapping phases, the acute or septicaemic phase and the immune phase [116]. Since then, the clinical presentation of the disease has been generally described as a biphasic

pattern with an initial acute or septicaemic phase. This is characterised by leptospiraemia which usually lasts for approximately seven days. Patients typically present with a sudden onset of febrile illness. The fever then subsides for few days before the immune phase begins. The immune phase is characterised by the production of antibody against *Leptospira* and excretion of leptospires in the urine (Figure 1.16) [98]. Most of the complications of leptospirosis are associated with localisation of leptospires within the tissues during the immune phase, starting the second week of illness. Fever can be biphasic and may reoccur in this phase [94, 98]. In addition to the biphasic pattern of fever, the clinical presentation of leptospirosis is also classified into two forms, based on the severity of clinical symptoms: anicteric and icteric leptospirosis [98].

Anicteric leptospirosis is the milder form of the disease. The vast majority of people infected with leptospires may be asymptomatic or have very mild symptoms with a self-limiting infection and do not seek medical care. Several serological studies in endemic areas have revealed evidence of subclinical infection. A population-based case-control study in Seychelles, the country with the highest incidence globally, reported that 37% and 9% of controls had past or current subclinical infection, respectively [117]. A smaller proportion of patients present with an acute febrile illness with other symptoms including chills, headache, myalgia, tiredness, abdominal pain, conjunctival suffusion, nausea, vomiting, diarrhoea, and less commonly, a skin rash [94, 98, 107]. These anicteric symptoms usually last for approximately a week and their resolution corresponds with the appearance of antibodies. The fever may be biphasic and may reoccur after a remission of a few days (Figure 1.16). The mortality is almost zero in anicteric leptospirosis. Few patients present with severe complications. Aseptic meningitis may be found in less than a quarter of cases and pulmonary haemorrhage may complicate the course of illness and lead to mortality [98].

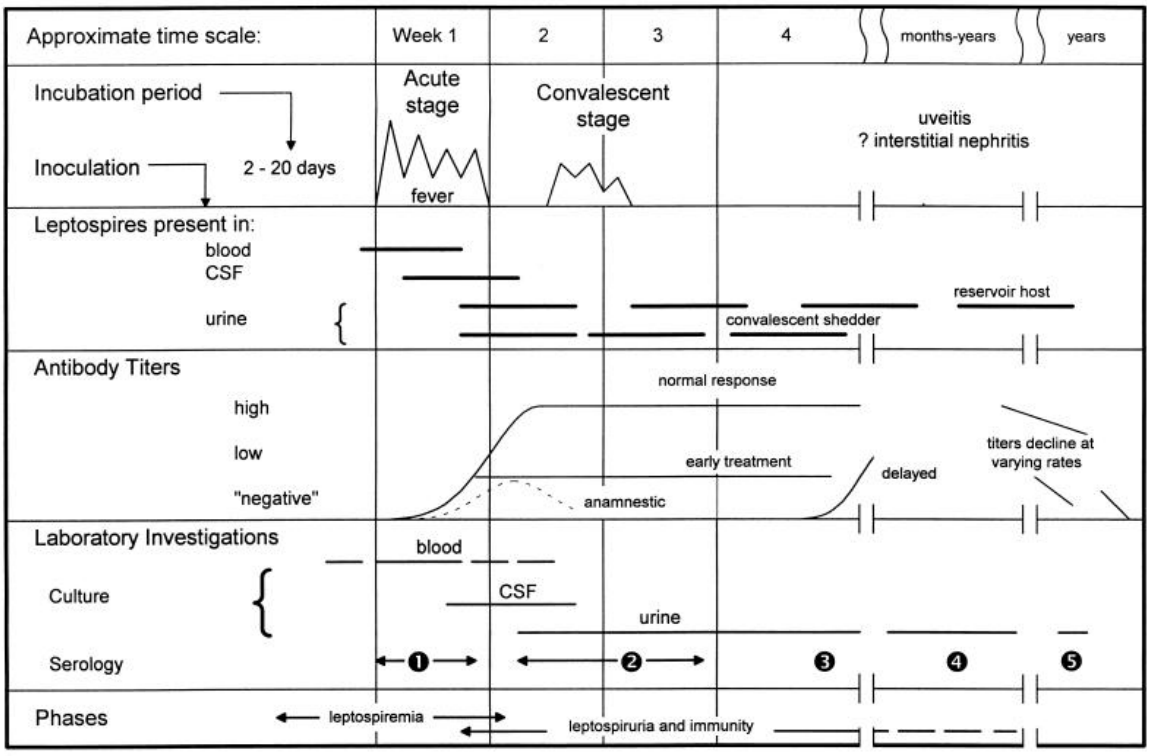


Figure 1.16 Biphasic nature of leptospirosis and relevant investigations at different stages of disease.

Note: Specimens 1 and 2 for serology are acute phase specimens, 3 is a convalescent phase specimen which may facilitate detection of a delayed immune response, and 4 and 5 are follow-up specimens which can provide epidemiology information, such as the presumptive infecting serogroup.

Reproduced from Levett, P.N., *Leptospirosis.* Clin Microbiol Rev, 2001. **14**(2): p. 296-326 with permission from American Society for Microbiology [98].

Icteric leptospirosis is a severe form of the disease. The clinical course of the icteric form often progresses very rapidly. This severe form occurs in between 5-10% of all patients with leptospirosis. Severe cases frequently present late in the course of their illness, which contributes to the high mortality rate (range between 5-15%) [98]. Severe leptospirosis is characterised by multiple organ dysfunction including liver, kidneys, lungs,

and brain [107]. Jaundice presents in this form of the disease. The combination of jaundice, renal failure and haemorrhage is known as Weil's disease, the most severe form of this disease. The syndrome can develop after the acute phase as the second biphasic phase, or the two phases may be not obviously distinguished and present as a single, progressive illness [94, 107].

1.3.4 Laboratory diagnosis of leptospirosis

The diagnosis of leptospirosis based on clinical presentation is difficult and inaccurate because of its broad spectrum and non-specific clinical features. Laboratory diagnosis is important for confirmation of disease status. Laboratory diagnosis may be achieved by the detection of the organisms or their components in clinical specimens i.e. isolation of leptospires in culture, detection of nucleic acid or detection of specific antibodies.

1.3.4.1 Microscopic examination

Dark-field microscopy (DFM) has been used to directly examine body fluids such as blood, cerebrospinal fluid (CSF), urine and dialysate fluid, but the test lacks both sensitivity and specificity. A study from India showed 60% sensitivity and 61% specificity when using DFM alone compared with IgM ELISA and clinical diagnosis [118]. Diagnosis of clinical specimens using this method has demonstrated that both false positive and false negative diagnoses can be easily made even in experienced hands [119].

1.3.4.2 Isolation and identification of *Leptospira*

Leptospiraemia occurs during the first stage of illness, beginning before the symptom onset and usually declines by the end of the first week of acute illness [98]. During this phase, the leptospires can be isolated from blood and CSF [94]. Urine culture could be performed from the beginning of the second week of symptom onset although the duration of urinary excretion varies, but may be several weeks (Figure 1.16) [94].

Definitive diagnosis could be made by the culture and isolation of *Leptospira* using the recommended Ellinghausen, McCullough, Johnson, and Harris (EMJH) medium [120]. A study from Thailand has reported that whole blood and deposit from spun plasma are the optimal specimens to use for culture [120]. However, isolation of leptospires by culture is rarely used as a diagnostic test in clinical settings because it is complicated, time consuming, labour intensive and has low sensitivity. Culture should be maintained for at least 12 weeks and requires weekly examination by the DFM before a negative result can be reported [115, 120]. A new solid medium, named *Leptospira* Vanaporn Wuthiekanun (LVW) agar, facilitates more rapid growth and enables isolation of a single colony and simple antimicrobial susceptibility testing of *Leptospira* spp.: clear minimum inhibitory concentrations (MICs) can be read within seven days after inoculation [121]. However, the LVW agar has never been evaluated as a diagnostic test and has not been used for primary diagnosis.

Leptospire isolates are identified either by serological or molecular methods. Traditional methods relied on CAAT, which only a small number of laboratories perform. Monoclonal antibodies are available for many serovars but not all. More recently, molecular methods for identification and typing of leptospires have been developed and studied extensively. The methods include PCR and digestion of chromosomal DNA by restriction endonucleases (PCR-REA), nested-PCR restriction fragment length polymorphism (PCR-RFLP), real-time PCR, pulse field gel electrophoresis (PFGE), loop-mediated isothermal amplification (LAMP), sequencing and many PCR-based methods [122-126]. Sequence-based identification of *Leptospira* is increasingly becoming standard. PFGE has been shown to identify most serovars. It is more complementary to rather than replacement of the serological methods. Whole genome sequencing will likely become the reference laboratory standard in the near future. Strain subtyping for epidemiological purposes can be performed by simple methods such as RFLP. However, this method has

poor reproducibility between laboratories. More recently sequence-based methods such as multiple locus sequence typing (MLST) have been developed (based on PCR technique and DNA sequencing) and applied to characterize different gene targets. This method has advantages over other typing methods. It is simple to standardise on an automated DNA sequencer and reproducible within and between laboratories. This method can be used for molecular epidemiological and evolutionary studies as well as population genetics [127, 128].

1.3.4.3 Serology-based diagnosis

In most cases, leptospirosis is diagnosed by serological methods because of limited capacity for culture and molecular diagnosis. IgM antibody is detectable in the blood at approximately 5-7 days and peaks at 2-3 weeks after onset of illness (Figure 1.16) [107]. An acute blood specimen for serology should be taken as soon as possible at presentation of the patient and a convalescent specimen should be taken at least a week later. An increased IgM antibody titre in the convalescent specimen compared with the acute specimen suggests acute or current infection. Serological methods can be divided into those in which the titre of antibody can be quantified such as MAT, IFA and ELISA, and those which can give only qualitative results (positive or negative) such as ICT and latex agglutination test (LA). In addition, serological diagnostic tests can also be classified into two main categories: serovar-specific (i.e. MAT) and non-serovar-specific tests (i.e. ELISA, IFA, ICT, LA) [107].

The use of agglutination tests was described soon after the first isolation of the organism when only a few serovars were recognised [98]. The “gold standard” reference method for confirmatory serological diagnosis of leptospirosis is the MAT, in which sera from patients are reacted with a suspension of live antigens from a panel of leptospiral serovars [108, 119]. However, this method requires the laboratory to maintain panels of

live leptospires, of which there are more than 250 serovars, based on a culture method which is time consuming and requires technical expertise. Repeated weekly subculture is also a hazard to laboratory workers [98]. MAT interpretation is complicated by a high degree of cross-reaction between different serogroups particularly in acute phase specimens. It is insensitive during the acute phase of illness, since antibody levels usually are not detectable before the first few days after infection. Like other serological methods, paired specimens are required to confidently confirm diagnosis. The cut-off titre is uncertain, depending on the background level of exposure in the population and seroprevalence, hence different cut-offs and interpretations are applied in different geographical localities [98, 129-132]. It is important to include a range of antigens used that represent all serogroups and locally common serovars, since the MAT is serogroup specific but not accurate to detect infecting serovar [107]. A recent study in Thailand evaluated MAT accuracy and indicated that this assay is a poor predictor of infecting serovar. Culture of infecting isolates and CAAT remain the technique of choice during epidemiologic studies in this setting [133].

Due to the limited ability to detect early stage of illness and complexity of the MAT, other serodiagnostic methods have been developed. Complement fixation (CF) was widely used, but the assay was not standardized and has been replaced by ELISA. There are many applications and modifications of ELISA as well as other serological tests including IgM ELISA assays [134], IgM dipstick assays [135], IgM Immunoblots [130], IgM dot-ELISA dipstick tests [136], IHA [137], LA [138], and lateral flow rapid tests [139]. These tests are simple, rapid, allow detection during acute phase of the disease and results can be obtained quickly enough for patient management. However, the overall conclusion of studies of these serological diagnostic tests is that the diagnostic accuracy in a clinical setting in endemic areas is poor and the tests are relatively expensive, especially in the rural areas of developing countries. ELISA tests are less specific than MAT since

they are based on genus-specific antigens which do not give an indication of the infecting serovars and these results require confirmation by MAT [108, 140]. Other serodiagnostic methods, including IFA [136, 141], radioimmunoassay (RIA) [142], counterimmunoelectrophoresis (CIE) [143, 144], and thin-layer immunoassay (TIA) [145], have been developed but are not widely used. The rapid bedside RDTs are inaccurate for early diagnosis of acute leptospirosis infection [110, 139, 146].

1.3.4.4 Molecular-based diagnosis

Leptospiral DNA has been amplified from various clinical specimen types such as serum, urine, CSF and a number of post-mortem organ specimens [107]. Many quantitative real-time PCR (qPCR) using TaqMan probe-based assays have been developed to detect leptospiral DNA and quantify the bacterial load in clinical and environmental specimens. Assays developed for diagnostic use can be considered in two main categories, targeting either housekeeping gene such as *rrs, gyrB,* or *secY,* or pathogen specific genes such as *lipL32*, *lig* or *lfbl* [107]. A *16S rRNA* gene qPCR has been developed and shown to be specific and able to discriminate pathogenic from non-pathogenic strains. The limit of detection of leptospires was as few as two cells in serum and 10 cells in urine [147]. A more recent qPCR assay was developed targeting the *LipL32* gene, which is present only in pathogenic strains. The lower limit of detection was 20 genomic equivalents (GE)/reaction. This study suggested that whole blood and plasma were better specimens compared to serum when testing spiked specimens [148]. These two assays, 16S *rRNA* and *LipL32* qPCRs have subsequently been evaluated in a clinical setting to use as a tool for early detection [149, 150]. The clinical sensitivity and specificity compared against positive culture and/or MAT using acute whole blood specimen were 56.0% (95% CI 47.0-64.0) and 90.0% (95% CI 83.0-94.0) for the 16S *rRNA* assay, and 43.0% (95% CI 34.0-52.0) and 93.0% (95% CI 88.0-97.0) for the *LipL32* assay, respectively. In addition, both assays showed high sensitivity in subgroup of culture positive patients with 95.0% (95% CI 83.0-

99.0) and 87.0% (95% CI 73.0-96.0) for 16S *rRNA* and *LipL32* assays, respectively. Early diagnosis of leptospirosis using qPCR is possible particularly when culture is not available [149]. LAMP is an inexpensive alternative method for rapid DNA detection. A *LipL41* LAMP was the first assay of this method for the detection of leptospires with a limit of detection of 100 GE/reaction [151]. Subsequently, a 16S *rRNA* LAMP was developed to detect pathogenic and intermediate leptospires. The limit of detection was 10 GE/reaction [125]. Clinical evaluation of the *LipL41* LAMP was also done in this study, using acute blood specimen compared to positive culture and/or MAT. The sensitivity and specificity of the 16S *rRNA* LAMP assay were 43.6% (95% CI 35.0-52.5) and 83.5% (95% CI 76.0-89.3), respectively, and for *LipL41* LAMP were 37.6% (95% CI 29.3-46.4) and 90.2% (95% CI 83.9-94.7), respectively. Similar to the qPCR assay, the sensitivity of the 16S *rRNA* LAMP assay was high in patients with positive culture (84.6% [95% CI 69.5-94.1]). The 16S *rRNA* qPCR and LAMP assays could detect around half of the patients with leptospirosis, but the clinical utility of LAMP assay was reduced by low specificity. Further evaluation of these assays in a clinical setting is still needed [125]. A limitation of PCR- or LAMP-based diagnostic assays is the current inability to identify the infecting serovar. Although this is not significant for individual patient management, it has great value for both epidemiological and public health aspects of disease management. However, whole genome sequencing has recently been applied to clinical specimens and it is possible that direct serovar identification may be possible in the future.

1.4 Rickettsial diseases

1.4.1 The Rickettsia and Orientia

Rickettsial diseases are zoonoses and are among the oldest known vector-borne diseases, caused by a variety of obligate intracellular Gram-negative bacteria of the order *Rickettsiales* and are vectored by arthropods and trematodes [152]. The bacteria in the order *Rickettsiales* have long been described as short, Gram-negative rods that retain basic fuchsin when stained by the Gimenez's method [153] and are strictly associated with eukaryotic cells. Historically, the order *Rickettsiales* included the families Rickettsiaceae, Bartonellaceae and Anaplasmataceae [154]. However, the classification and taxonomy of *Rickettsiales* has undergone major reorganisation in the past decades. Traditionally, members of the order *Rickettsiales* were classified based on morphological, antigenic, and metabolic characteristics. The classification has been continually modified as more data have become available, particularly the new identification techniques based on molecular methods and phylogenetic studies, especially 16S *rRNA* gene sequence analyses. For example, members of the family Bartonellaceae (including *Bartonella quintana,* causative agent of trench fever), although highly fastidious, are cultivable on enriched bacteriological culture media and phylogenetically lie in the alpha 2 subgroup of the *Proteobacteria*, unlike other *Rickettsia* species that are in the alpha subgroup. These 16S *rRNA* sequences resulted in removal of the family Bartonellaceae from the order *Rickettsiales* [155]. Furthermore, C*oxiella burnetii,* causative agent of Q fever, has also been removed from the *Rickettsiales* because its 16S *rRNA* sequence was more similar to members of the gamma subgroup of the *Proteobacteria* [155]. Hence, they were removed from this order which currently comprises two families: Anaplasmataceae and Rickettsiaece [156]. The order *Rickettsiales* includes bacterial members of the genera *Anaplasma, Ehrlichia, Neorickettsia, Orientia, Rickettsia* and *Wolbachia* [157]. The diseases caused by these organisms are commonly classified into three main groups

including rickettsioses caused by bacteria of the genus *Rickettsia*; scrub typhus caused by the genus *Orientia*, both from family Rickettsiaceae; and ehrlichioses and anaplasmoses caused by bacterial members of the family Anaplasmataceae [158].

Rickettsiae are obligate intracellular Gram-negative bacteria that can be visualised by Gimenez and Giemsa stains, but not Gram stain. Cell size ranges from 0.3x0.8μm to 0.5x2.0μm [159]. Currently, the genus *Rickettsia* contains at least 27 recognised species and several dozens of uncharacterised strains [160]. *Rickettsia philipii, Rickettsia monacensis* and *Rickettsia hoogstraalii* were recognised in addition to the 25 validated species that previously reported since 2009 [157]. The members of the genus *Rickettsia* are classified into (1) spotted fever group (SFG) rickettsiae that included the largest number of species (more than 20 different species); (2) typhus group (TG) rickettsiae that contains *Rickettsia prowazekii* and *Rickettsia typhi*; (3) the *Rickettsia bellii* group and (4) the *Rickettsia canadensis* group. The *R. bellii* and *R. canadensis* have not so far been reported to cause human infection [152]. Table 1.3 summarises the characteristics of validated *Rickettsia* species. The scrub typhus group (STG) of *Orientiae* currently includes *Orientia tsutsugamushi*, *Orientia chuto* and potentially other Orientia and Orientia-like spp. [161-163].

The genome sizes of *Rickettsiales* are small as a result of reductive evolution and consist of a single circular chromosome. The genome sequences of more than ten species within *Rickettsiales* are available and the sizes range from 0.8 to 2.1mb [164]. There are some bacteria that have highly stable genome without virtually repeats or mobile elements such as *R. prowazekii* (genome size 1.1mb) [165], whereas others have an extraordinarily high proportion of repeats and conjugative elements such as *O. tsutsugamushi* (genome size 2.1mb), its repeat density is 200-fold higher than that of *R. prowazekii* [166]. Currently, there are 45 strains within *Rickettsia* spp. that have genome sequences available.

These sequences reveal wide variations in size and genome content. The genome sizes vary from 1.11 to 2.1mb for *R. typhi* and *R. prowazekii*, and a *Rickettsia* endosymbiont of *Ixodes scapularis*, respectively [160].

This thesis focuses on the family Rickettsiaceae, including *Rickettsia typhi* and *O. tsutsugamushi,* as they are currently considered the major rickettsial infections in Thailand [7, 167]. *R. typhi* is small (0.4x1.3μm). It is the causative agent of murine typhus, also known as endemic typhus or flea-borne typhus [168]. *O. tsutsugamushi* was formerly known as *Rickettsia tsutsugamushi.* The name *Orientia* is from Orient, the area where this organism is widely distributed [161]. The *Orientia* is an obligate intracellular Gram-negative bacterium that propagates in host cell cytoplasm by binary fission. The cell size is approximately 0.5-0.8μm in diameter and 1.2-3.0μm long, which is slightly larger than that of *R. prowazekii* (the causative agent of epidemic typhus)*, R. typhi* (the agent of murine typhus) and *Rickettsia rickettsii* (the agent of Rocky Mountain spotted fever, RMSF) [169]. *O. tsutsugamushi* has various antigenic variations. Hence, many different antigenic types of strains exist including the three major types that are well known, Gilliam, Karp and Kato. Other antigenic types such as Shimokoshi, Kawasaki, Kuroki and others have also been described [161]. The distribution of the strains is dependent on the geographical area.

Table 1.3 Characteristics of validated *Rickettsia* species.

Species	Type strain	Vector	Geographical distribution	Pathogenic role
R. aeschlimannii	MC16	*Hy. marginatum, R. appendiculatus, Ha. punctata*	France, Morocco	Unnamed spotted fever
R. africae	ESF-5	*Am. hebraeum, Am. variegatum, R. appendiculatus*	Sub-Saharan Africa, Reunion Island, West Indies	African tick-bite fever
R. akari	MK	*Al. sanguineus*	USA	Rickettsialpox
R. asiatica	IO-1	*I. ricinus*	Japan	Unknown
R. australis	Phillips	*I. holocyclus, I. tasmani, I. cornuatus*	Australia	Queensland tick typhus
R. bellii	369L42–1	*D. variabilis, D. occidentalis, D. albopictus, Ha. lepopalustris, Am. cooperi, Ornithodoros concanensis, Argas cooleyi*	USA, Brazil	Unknown
R. canadensis	2678	*Ha. Leporispalustris*	USA	Unknown
R. conorii subsp.conorii	Malish 7	*R. sanguineus, Ha. Leachii*	Mediterranean area, Africa	Mediterranean spotted fever
R. conorii subsp. Indica	ITTR	*R. sanguineus, B. microplus, Ha. leachii*	India	Indian tick typhus
R. conorii subsp. caspia	A-167	*R. sanguineus, R. pumilio*	Chad, Kosovo, Russia	Astrakhan fever
R. conorii subsp. israelensis	ISTTCDC1	*R. sanguineus*	Israel	Israeli spotted fever
R. felis	URRWXCal2	*Ctenocaphalides felis, Ar. erinacei*	Worldwide	Flea spotted fever
R. heilongjiangensis	54	*D. silvarum*	China, Russia, Thailand	Far Eastern rickettsiosis
R. helvetica	C9P9	*I. ricinus, I. ovatus, I. persulcatus, I. monospinosus*	Europe, Japan	Suspected agent of a rickettsiosis
R. honei	RB	*Ap. Hydrosauri, Am. cajennense, I. granulatus*	Australia	Flinders Island spotted fever
R. japonica	YH	*D. taiwanensis, Ha. flava I. ovatus, H. longicornis*	Japan	Oriental or Japanese spotted fever
R. massiliae	Mtu1	*R. sanguineus, R. turanicus, R. mulsamae, R. lunulatus, R. sulcatus*	France	Unnamed rickettsiosis
R. montanensis	M/5–6	*D. variabilis, D. andersoni*	USA	Unknown

Table 1.3 Characteristics of validated *Rickettsia* species (continued)

Species	Type strain	Vector	Geographical distribution	Pathogenic role
R. parkeri	Maculatum20	*Am. maculatum, Am. triste, Am. americanum*	USA	Unnamed rickettsiosis
R. peacockii	Skalkaho	*D. andersoni*	USA	Unknown
R. prowazekii	Breinl	*Pediculus humanus humanus*	Africa, Russia, South America	Epidemic typhus
R. raoultii	Khabarovsk	*R. pumilio, D. nttalli, D. marginatus, D. Silvarum, D. reticulates*	France, Russia	TIBOLA or DEBONEL
R. rhipicephali	3–7-6	*D. occidentalis, R. sanguineus*	Africa, Europe, USA	Unknown
R. rickettsii	Sheila Smith	*Am. aureolatum, Am. cajennense, D. andersoni, D. variabilis, R. sanguineus*	Brazil, Mexico, Panama, USA	Rocky mountain spotted fever
R. sibirica subsp. Sibirica	246	*D. nuttalli, D. silvarum, D. marginatus, D. auratus, D. sinicus, D. pictus, Ha. concinna, Ha. yeni, Ha. Wellingtoni*	China, Russia	Siberian or North Asian tick typhus
R. sibirica subsp. Mongolitimonae	HA-91	*Hy. asiaticum, Hy. Truncatum*	Algeria, China, France, Greece, South Africa	Lymphangitis-associated rickettsiosis
R. slovaca	13-B	*D. marginatus, D. reticulates*	Europe, Russia	TIBOLA or DEBONEL
R. tamurae	AT-1	*Am. Testudinarium*	Japan	Unknown
R. typhi	Wilmington	*X. cheopis, C. felis, L. segnis*	Worldwide	Murine typhus

Al.=Allodermanyssus; Am.=Amblyomma, Ap.=Aponomma; Ar.=Archeopsylla; B.=Boophilus; D.=Dermacentor; Ha.=Haemaphysalis; Hy.=Hyalomma; I.=Ixodes; L.=Leptopsylla; R.=Rhipicephalus; X.=Xenopsylla

DEBONEL=*Dermacentor*-borne necrosis and lymphadenopathy; TIBOLA=Tick-borne lymphadenopathy

This table is reproduced with permission from Fournier, P.E. and Raoult, D., *Current knowledge on phylogeny and taxonomy of Rickettsia spp.* Ann N Y Acad Sci, 2009. 1166: p. 1-11. Copyright John Wiley and Sons [157].

1.4.2 Epidemiology of Rickettsia and Orientia

1.4.2.1 Transmission

The epidemiology of human diseases caused by rickettsial infections is closely related to their vectors, natural hosts and their geographic distribution as shown in Table 1.3. The organisms are associated with arthropods including ticks, mites, fleas, lice, and mosquitoes, which may act as vectors or reservoirs [160]. The SFG rickettsiae are transmitted mainly by *Ixodidae* (hard ticks), but also transmitted by *Argasidae* (soft ticks; i.e. *Rickettsia hoogstraalii*), fleas (*R. felis*) and mites (*R. akari*). The TG rickettsiae are transmitted by human body lice (*R. prowazekii*) and fleas (*R. typhi*).

Murine typhus is transmitted by fleas, in which the main vector is rat fleas (*Xenopsylla cheopis*), and the main reservoirs are rodents, mainly *Rattus norvegicus* and *Rattus rattus.* However, many other rodents, as well as wild and domestic animals, including cats, are also hosts. The transmission occurs by contact with flea feces that are contaminated with *R. typhi* during or after blood feeding, either by inhalation, conjunctival contamination or disrupted skin or by flea bites [160]. In the majority of cases, transmission is associated with areas that have a large number of rats around. Fleas are also the vectors of other rickettsiae such as *R. felis*, mainly cat flea (*Ctenocephalides felis*). Arthropods can acquire the organisms by vertical transmission or by co-feeding, occurring when several arthropods feed next to one another on the same host. These may complicate the transmission and epidemiology of rickettsiae [160].

Scrub typhus is transmitted by the bite of the infected larval stage of the trombiculid mite, called chigger. Its primary vector is *Leptotrombidium deliensis,* but several other species are also implicated [170]. Humans are accidental dead-end hosts that become infected when residing in areas that have infected chiggers: person-to-person transmission has never been reported. Infection is mainly associated with active rice field

activities, but many cases are acquired during other outdoor activities, for example logging and military operations. *O. tsutsugamushi* is maintained in mites by highly efficient transovarial transmission (from females to their offspring via eggs). The transmission of *O. tsutsugamushi* appears to be dependent on seasonal activities between chiggers and humans, during the seasons of high and stable temperature and humidity [167]. A study from Thailand confirmed this seasonal variation, by demonstrating that most patients were diagnosed with scrub typhus during the end of the rainy season and the beginning of winter, whereas murine typhus was evenly diagnosed in all seasons [170].

1.4.2.2 Global distribution and incidence

Geographical distribution of *Rickettsia* and *Orientia* largely depends on the arthropod vectors and reservoirs, since most of these vectors prefer specific optimal environmental conditions and hosts [167]. The global distribution of *Rickettsia* and *Orientia* are shown in Figure 1.17 and Figure 1.18. Murine typhus is an urban disease. It has a worldwide distribution, whereas scrub typhus occurs mainly in rural areas and is endemic in the Asia Pacific region. Scrub typhus and murine typhus are the most common rickettsial causes of acute undifferentiated fever and are widely endemic in Asia, but a number of SFG rickettsia have also been recognized in Asia including *R. felis* [7, 156, 170-174] and *R. honei* [175]. Twelve cases of *R. felis* infection have been reported in Asia; three from Thailand [171, 176], three from Republic of Korea [177], three from Sri Lanka [178], one from Laos [172], one from Taiwan [179] and one from Israel [180].

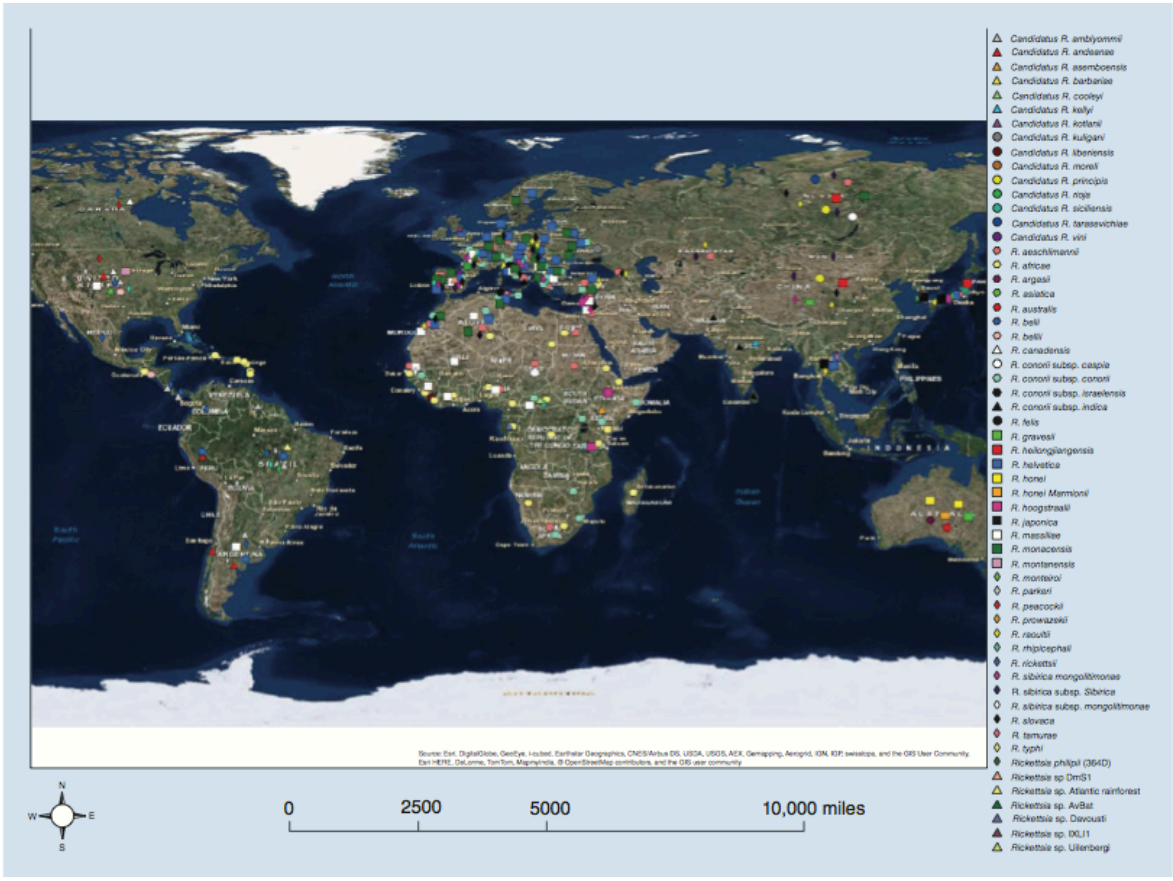


Figure 1.17 Worldwide distribution of *Rickettsia.* World map detailing the presence of selected rickettsial species (as indicated by symbols) by continent/country.

Reproduced from Luce-Fedrow, A., Mullins, K., Kostik, A.P., St John, H.K., Jiang, J., and Richards, A.L., *Strategies for detecting rickettsiae and diagnosing rickettsial diseases.* Future Microbiol, 2015. 10(4): p. 537-64 with permission from Future Medicine Ltd [152].

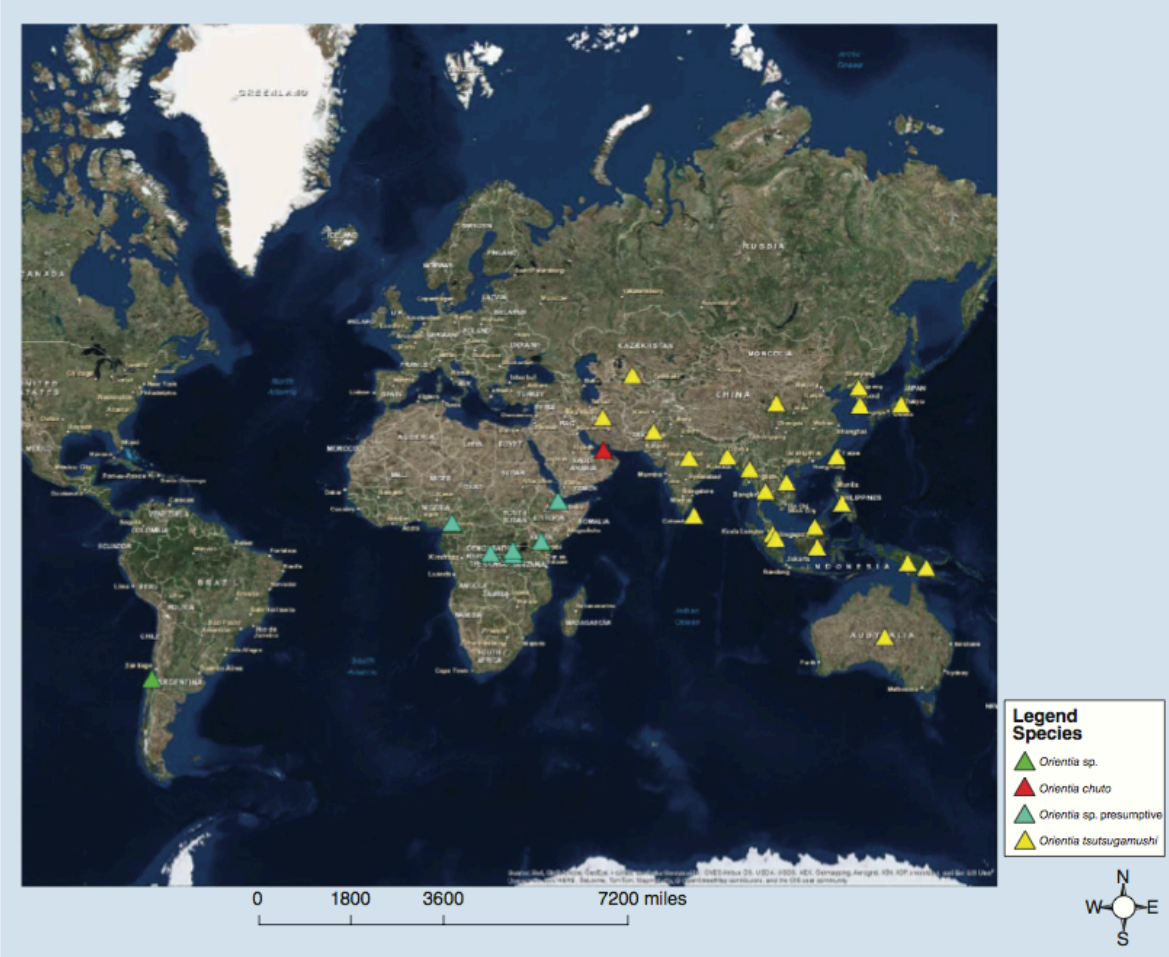


Figure 1.18 Distribution of *Orientia.* World map detailing the presence of selected *Orientiae* (as indicated by symbols) by continent/country.

Reproduced from Luce-Fedrow, A., Mullins, K., Kostik, A.P., St John, H.K., Jiang, J., and Richards, A.L., *Strategies for detecting rickettsiae and diagnosing rickettsial diseases.* Future Microbiol, 2015. 10(4): p. 537-64 with permission from Future Medicine Ltd [152].

1.4.2.3 Scrub typhus and murine typhus in Thailand

There is an estimated one billion people at risk and one million cases of scrub typhus occurring worldwide each year [167]. Scrub typhus and murine typhus are widespread in Thailand. Although, the data on incidence of murine typhus is scarce there are individual reports from studies indicating that murine typhus is circulating [170].

In Thailand, less than 100 scrub typhus cases were reported per year before 1983, 750-900 cases per year were reported between 1988 and 1991, and 3,000-5,000 cases per year have been reported since 2001 [170]. The most up to date data for scrub typhus from the Centre of Epidemiological Information, Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand is up until 2014. There were approximately 4,000-11,000 scrub typhus cases reported each year between 2003 and 2014, with morbidity rates ranging from 5.98 to 17.09 per 100,000 population. The number of scrub typhus cases and morbidity rate have been increasing in recent years and peaked in 2013 (Figure 1.19). The number of deaths and mortality rate varied over the same period but remained below 10 cases a year with a maximum mortality rate of 0.02/100,000 population in 2012 (Figure 1.20). For reasons discussed previously, the scrub typhus mortality rate is unreliable due to the routine surveillance system approach.

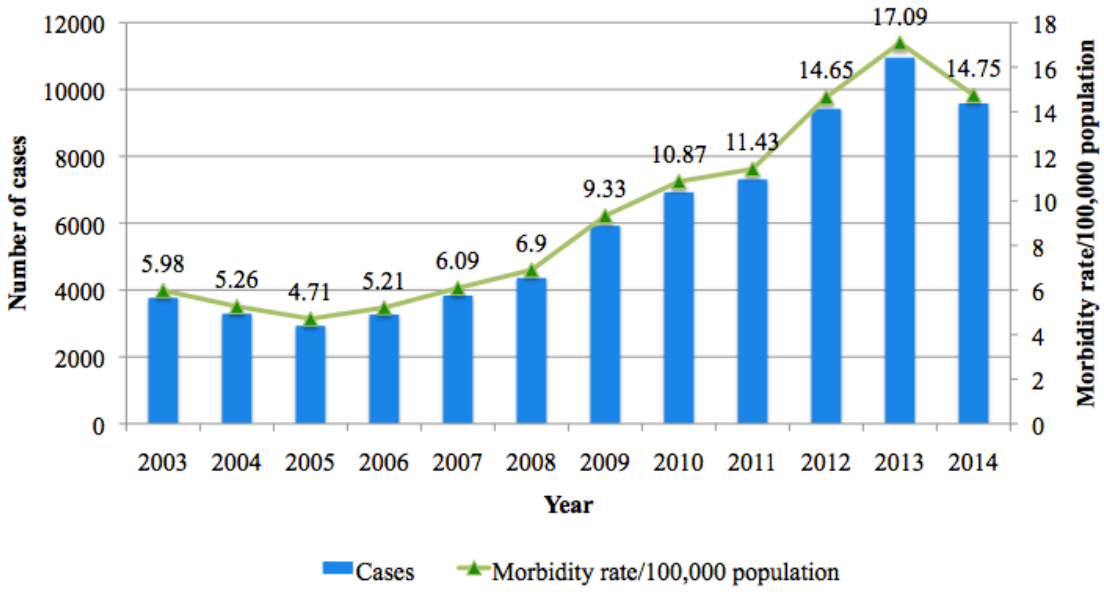


Figure 1.19 Number of cases and morbidity rate of scrub typhus reported in Thailand between 2003-2014.

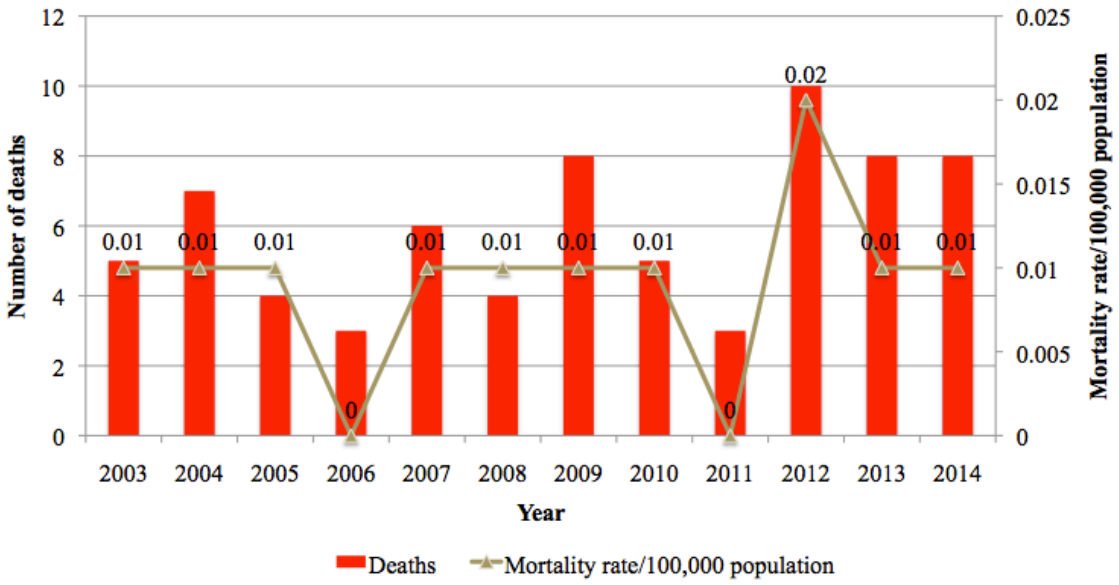


Figure 1.20 Number of deaths and mortality rate of scrub typhus reported in Thailand between 2003-2014.

Source for Figure 1.19 and Figure 1.20: Centre of Epidemiological Information, Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand, http://www.boe.moph.go.th/boedb/surdata/disease.php?dcontent=old&ds=44.

There are no summary statistical data available for murine typhus from the national disease surveillance report from the Bureau of Epidemiology, Thailand.

1.4.3 Clinical features of scrub typhus and murine typhus

Rickettsial infections are difficult to diagnose even by experienced health care providers. The early symptoms and signs of these infections are notoriously non-specific. Diagnosis of scrub typhus and murine typhus based on clinical presentations is difficult as clinical symptoms and signs of these diseases overlap with those of other acute febrile illnesses such as other rickettsial infections, leptospirosis and dengue [7].

Scrub typhus

Scrub typhus is an acute febrile illness. Its clinical manifestations range from a mild and self-limiting illness to a more severe, and sometimes fatal, disease. The general course and prognosis of the disease vary according to the characteristics of the endemic strain. The classical clinical manifestation of scrub typhus usually consists of, but is not limited to, eschar, regional and generalised lymphadenopathy, and maculopapular rash [167, 181]. After infection, clinical symptoms appear after an incubation period of 5-20 days [181]. Fever in scrub typhus rises abruptly and peaks in days 3-4 of the disease. The fever can persist for more than three weeks in untreated patients [181]. The primary clinical presentations are eschar (often not present) and regional lymphadenopathy followed by fever, headache, myalgia, generalised lymphadenopathy, cough, gastrointestinal symptoms, transient hearing loss and rash [182]. Complications may develop after the first week of illness. Meningoencephalitis, pulmonary involvement, acute renal failure, and disseminated intravascular coagulation, may manifest in severe cases [167, 181].

The chigger bite site can appear on any part of the body, however it often localised in the areas that are difficult to examine, such as the genital region or under the armpit. The bite sites are usually painless and not recognised by the patients. This site may later develop into an eschar. It begins as small papule and enlarges with a central black necrosis resembling the mark of a cigarette burn [167]. A single eschar is usually found, although more than one infected chigger can attack a patient resulting in multiple eschars [183, 184]. A prospective study from Korea reported a differential distribution of eschars in males and females among 176 scrub typhus patients. An eschar was present in 92% of the patients, including one patient with three eschars. The presence of eschar was more common on the front of the body rather than on the back for both genders. For males, the

most common site was the area within 30 centimetres below the umbilicus, whereas the front chest above the umbilicus was the most common in females [184].

The eschar can be seen early in the course of disease: they are usually formed by the time that fever appears and can be a useful clinical diagnostic clue, although their presence is widely variable ranging from 7-97% [182, 185, 186]. In some cases, an atypical eschar can be found in warm and damp areas such as the axilla or perineum, in which the necrotic eschar is not formed but instead, an ulcer with a shallow, purulent base surrounded by a clear, erythematous band may be formed. The eschar may be easily overlooked and misdiagnosed in these cases [184].

A transient maculopapular rash may appear at the end of the first week of illness, and last a few days to a week. The rash is often difficult to observe and usually predominates on the trunk and may appear on the face, palms and soles in rare cases [181].

Murine typhus

Murine typhus is a mild disease with non-specific symptom and signs. Although clinical features of murine typhus are generally mild or self-limited without complications in most cases, some patients develop septic shock, acute respiratory failure and multi-organ failure leading to death [187]. After an incubation period of 7-14 days, the most common clinical presentations include fever, headache, rash and arthralgia. Fever in murine typhus can last 3-7 days. Although rash is considered a common hallmark of rickettsial disease, it is neither always seen at presentation nor in all patients. The presence of rash in patients with murine typhus is widely variable ranging from 20-80% [168]. A study of scrub typhus and murine typhus from North Vietnam reported rash was more likely to be found in patients with murine typhus, whereas eschar was more significantly associated in patients with scrub typhus, and rarely seen in patients with murine typhus [188].

The rash may appear around one week after onset of fever and lasts for 1-4 days. It may be non-pruritic, macular, or maculopapular, usually occurs on the trunk and then spreads peripherally, sparing palms and soles [168]. Other clinical presentations have also been reported such as nausea, vomiting, abdominal pain, diarrhoea, jaundice, and cough. These various non-specific symptoms can lead to misdiagnosis at presentation. The clinical signs may last for 7-14 days in untreated patients [167].

1.4.4 Laboratory diagnosis

Clinical manifestations of rickettsial infections are widely variable and non-specific, thus clinical diagnosis at the time of presentation is difficult. Laboratory diagnosis is useful for confirmation of these infections. Understanding the time course of infection is crucial in diagnosing the disease.

During inoculation, the number of rickettsiae is too low to be detected in blood and humans do not develop disease at this stage. In scrub typhus and several SFG rickettsioses, eschar may develop at the inoculation site within a few days. At this stage, eschar can be used as a clinical specimen for laboratory diagnosis as the number of rickettsiae is multiplied and can be detected by culture, molecular methods or immunohistochemistry staining [189]. Figure 1.21 demonstrates detection and time course of rickettsial infections.

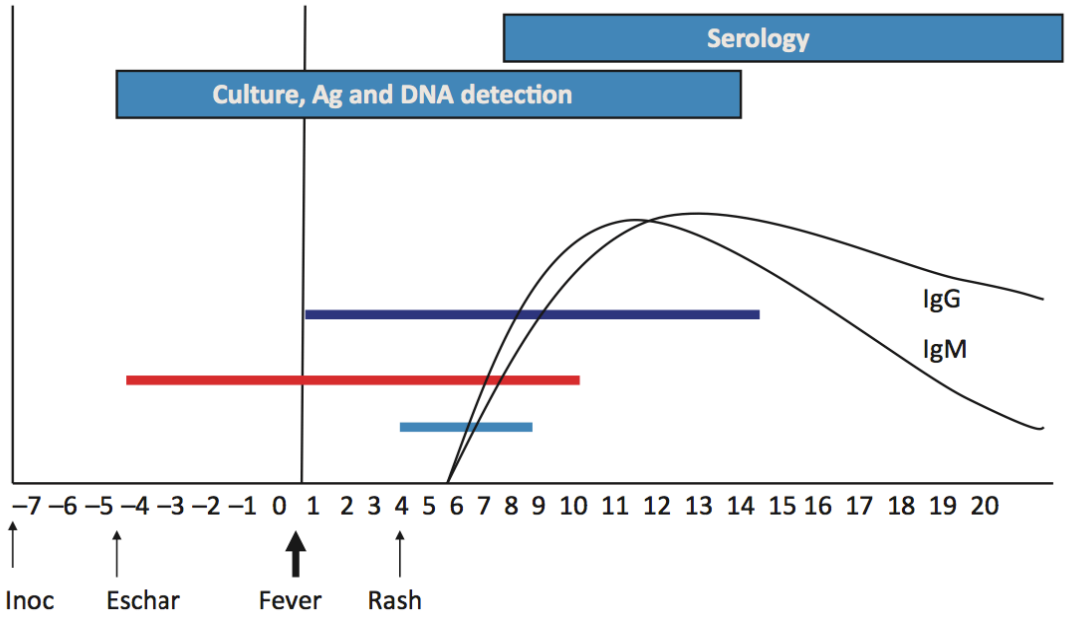


Figure 1.21 Detection and time course of rickettsial infections.

Ag= antigen, dark blue bar= fever, Inoc= inoculation, light blue bar= rash, red bar= eschar

Reproduced from Richards, A.L., *Worldwide detection and identification of new and old rickettsiae and rickettsial diseases.* FEMS Immunol Med Microbiol, 2012. 64(1): p. 107-10 with permission from Oxford University Press [189].

Blood specimens can be collected for the diagnosis when patient presents with fever and other clinical symptoms and signs. Duration of rickettsaemia varies by the organisms and by the use of antibiotics. Rash appears approximately 3-5 days after disease onset and lasts for approximately 5-8 days. Biopsy of a rash area can be used to detect rickettsiae. Culture of rickettsiae, antigen or DNA detection can be performed prior to disease onset, in the case of eschar presenting. However, it is more likely to be performed at the time of disease onset. Detection of IgM and IgG antibodies against rickettisiae in serum or plasma by serology methods are frequently used. In initial infection, IgM antibody generally appears by eight days after disease onset and increases rapidly, while IgG antibody does not appear until 12 days after disease onset and increases more slowly.

In re-infection, IgG antibody initially appears by six days, while IgM antibody is variably detectable [190]. The IgM and IgG antibody persist for months to years, respectively. The acute specimen should be collected as early in the course of disease as possible and the convalescent specimen should be collected at least 14 days after. Paired acute and convalescent specimens should be used to confidently confirm the diagnosis and a seroconversion or a fourfold rise in titre indicates a current infection [189, 191]. A single acute specimen can be used in settings where an optimal cut-off titre is already determined [192].

1.4.4.1 Staining and culture of Rickettsiae

Stains are not commonly used in term of diagnostics since they lack sensitivity and specificity, however the stains such as Giemsa, Gimenez, and acridine orange are still used in the research laboratory to monitor the presence of rickettsiae in cell culture [152].

Like other bacteria, isolation of the organisms is the most definitive method for diagnosis. However, culture of *Rickettsia* and *Orientia* is difficult as they are obligate intracellular bacteria that require a living host to grow. Isolation of these organisms has been performed using embryonated hen's eggs, Vero (African green monkey kidney) cells and L929 (mouse fibroblast) cells [193, 194]. Isolation can be performed from blood and skin biopsy. Shell vial cell culture assay remains the best tool for isolation of intracellular bacteria [193]. However, the sensitivity of culture is low. Recent analysis using Bayesian latent class models (LCMs) showed that blood culture for *O. tsutsugamushi* had sensitivity of 24.4% (95% CI 12.2-41.3) [195]. Moreover, culture is insensitive when antibiotic treatment has been given and specimens for culture should be inoculated as soon as possible to increase the sensitivity. The incubation time for culture positivity can be as long as 27 days (range 16-37) [194] and containment in biosafety level 3 laboratory facilities is required. Therefore, current methods of isolation are not appropriate for routine

diagnosis [196]. However, the culture method is essential for drug sensitivity testing, molecular epidemiology studies and for providing a source material for other diagnostic test evaluations.

1.4.4.2 Serology-based diagnosis

Similar to dengue and leptospirosis, serological tests are the most widely used diagnostic tools for rickettsial infections. Many serology-based diagnostic tests have been developed and have encountered the same issues found in the other diseases regarding the interpretation and diagnostic accuracy of the tests.

The Weil-Felix agglutination test is based on the detection of antibodies to various *Proteus* species that contain antigens which cross-react to antigens from *Rickettsia* spp. *Proteus vulgaris* strain OX-2 antigen cross-reacts with SFG rickettsiae except for RMSF. *P. vulgaris* strain OX-19 antigen cross-reacts with TG rickettsiae and RMSF, and *Proteus mirabilis* Kingsbury strain antigen (OX-K) cross-reacts with *O. tsutsugamushi* [197]. The test is easy to perform and inexpensive, and has long been widely used for diagnosis of rickettsial diseases. However, it has been replaced by other serological diagnostic assays because of lack of sensitivity and specificity [197-201].

The IFA and indirect immunoperoxidase test (IIP) have been widely used as reference serological methods to confirm the diagnosis using acute and convalescent sera. However, the tests are time consuming, require a fluorescence microscope (IFA) and experienced laboratory technicians. Cross-reactivity, variation of sensitivity and specificity depending on species and cut-off level determined by each laboratory remain problematic [159, 191, 196]. Within the same laboratory, the reading of the assays are inherently subjective if performed by different operators as showed in a study comparing inter- and intra-operation variability in reading the IFA for diagnosis of scrub typhus and murine typhus. The inter-operator variability was significantly related to the experience of the

operator in reading the tests [202]. Recent analysis using Bayesian LCMs to determine the optimal cut-off titre for IgM IFA in diagnosis of scrub typhus was assessed. The result demonstrated that the optimal cut-off titres of ≥1:3,200 in an acute specimen or of a ≥4-fold rise to ≥1:3,200 in a convalescent specimen provided the highest diagnostic accuracy (81.6% sensitivity and 100% specificity) [192]. However, further evaluation using this cut-off in other settings is required.

ELISAs have been developed to overcome the cross-reactivity amongst members of the genus *Rickettsia* by using blocking binding of monoclonal species-specific epitopes [203] or recombinant protein antigens [204, 205]. Evaluation studies of IgM capture ELISA using recombinant protein have revealed good performance and it may be able to replace IFA in a diagnostic laboratory, especially in rural areas where the complicated techniques are usually not available [204, 206]. Nevertheless, in disease endemic areas, these serodiagnostic assays require paired specimens to detect a rising titre of IgG in secondary infections [205]. This results in delayed diagnosis and therefore may not help patient management. A recent study evaluated an IgM antibody-based ELISA for acute diagnosis of scrub typhus and proposed to use it as an alternative reference test to the IgM antibody-based IFA. With the optimal cut-off optical density (OD) of 1.474 at a specimen dilution of 1:400, this IgM ELISA had high sensitivity and specificity using paired specimens (85.7% (95% credible interval [CrI] 77.4-86.7) sensitivity and 98.1% (CrI 97.2-100) specificity) [207].

Other antibody-based point-of-care tests have been developed for rapid diagnosis of scrub typhus and murine typhus, including ICT and immunoblot assays. In a study from Laos, IgM antibody-based ICT for scrub typhus and immunoblot assay for murine typhus were assessed on acute serum specimens. The sensitivity of the scrub typhus ICT was low, 23.8% (95% CI 15.9-33.3) when compared against a four-fold rising IgM IFA titre, and

39.1% (95% CI 34.1-44.2) when compared against an acute specimen IgM IFA titre of ≥1:400. However, the specificity was high for both comparisons, 86.2% (95% CI 84.1-88.6) and 99.5% (95% CI 98.7-99.9), respectively. For the murine typhus immunoblot assay, similarly, the test had low sensitivity but high specificity. The sensitivity was 61.2% (95% CI 53.7-68.3) when compared to a four-fold rising IgM IFA titre, and 54.6% (95% CI 49.1-60.0) when compared to an acute specimen IgM IFA titre of ≥1:400. The specificity was 86.5% (95% CI 84.1-88.8) and 94.1% (95% CI 92.0-95.7), respectively [208]. The antibody-based rapid tests generally have good specificity but poor sensitivity, therefore the usefulness of this test in clinical settings for acute diagnosis is limited. The use of total antibody-based rapid tests in disease endemic areas is a drawback rather than useful to the clinicians to differentiate acute infection from past infection. In such areas, patients are likely to have had previous infections, resulting in detection of the persistent IgG antibody responses and the test may have an inevitably low specificity [209].

1.4.4.3 Molecular-based diagnosis

In recent years, molecular assays have been developed and evaluated as rapid diagnostic tools for acute rickettsial infection. PCR assays have been developed for detection of Ricksettsial species and *O. tsutsugamushi* from blood and skin biopsy specimens and can detect the presence of organisms 4-8 days prior to the development of antibodies that are needed for serological testing [210]. Eschar and swabbed lesions or rash biopsies contain the highest number of organisms and are the specimen of choice. The sensitivity of PCR, to detect *O. tsutsugamushi,* can be as high as ~90% when using buffy coat or eschar specimens [211, 212]. However, using buffy coat specimen, PCR can be affected by antibiotic treatment, with sensitivity decreasing to 60.5% within three days after treatment and to 10.0% after four days of treatment [211]. Using eschar specimens, PCR remains positive for *O. tsutsugamushi* after treatment with antibiotics [212, 213].

1.4.4.3.1 Conventional PCR

Nested-PCR for detection of nucleic acid from acute blood specimens based on a gene encoding the 56kDa outer membrane protein (56kDa) has proven useful for early diagnosis of scrub typhus [214]. A prospective study from Korea proved the usefulness of this nested-PCR targeting 56kDa (slightly modified from Furuya *et al*., 1993 [214]) from buffy coat as a rapid and reliable test for confirming the diagnosis of the disease with 82.2% sensitivity and 100% specificity [215]. In addition, another study of the clinical usefulness of eschar PCR (56kDa) for diagnosis of scrub typhus was reported with high sensitivity and specificity (86.0% and 100%, respectively) [212]. However, in Northeast Thailand, it has been shown that this 56kDa PCR had a high specificity (99.2%) but a low sensitivity (29%). The low sensitivity may be explained by the sequence variability in the region of primer annealing as *O. tsutsugamushi* shows a high degree of genetic polymorphism [216].

1.4.4.3.2 Real-time PCR

A 16S *rRNA* gene SYBR Green-based real-time PCR assay was developed and evaluated for clinical diagnostic accuracy in a study of scrub typhus in Thailand. The assay had 44.8% sensitivity and 99.7% specificity compared against paired serology by IFA. This was considered insufficient to diagnose scrub typhus in this endemic setting [216].

Several specific and sensitive qPCR assays were established to enable quantification of bacterial loads in clinical specimens. They were able to detect <10 copies of purified target DNA [210, 217-220], however in clinical specimens the assays may not be as sensitive. Detection of the 16S *rRNA* gene by qPCR was reported to determine the *O. tsutsugamushi* loads in patients with scrub typhus. The analytical performance was high with 100% specificity and the limit of detection was 0.04 copies/µl but the clinical diagnostic accuracy was not determined [221]. A qPCR targeting a 47kDa outer membrane

protein gene highly specific for *O. tsutsugamushi* was developed and the assay was able to detect the DNA target at between 3-10 copies/reaction. The assay was tested in animal models during the development phase and was able to detect 3-21 copies/µl of monkey blood [210]. A study from Thailand evaluating this 47kDa qPCR assay to detect *O. tsutsugamushi* in patients with scrub typhus reported that the assay correctly detected 10/10 patients who were culture positive and could detect 7/17 patients who were culture negative. The *O. tsutsugamushi* loads ranged from 1,076 to 28,812 copies/µl of blood [222]. This assay will require further evaluation in clinical specimens. A qPCR assay targeting a 17kDa antigen gene (*Rickettsia*-specific) was developed to detect *Rickettsia* spp. in ticks. The limit of detection was found to be three copies/reaction [217, 223]. Another qPCR assay targeting the outer membrane protein B gene (*ompB*) was established for the detection of *R. typhi* in fleas. The detection limit was three copies/µl [218]. The 47kDa, 17kDa and *ompB* gene targets are widely used for research purposes and applied for different PCR formats [224-226]. However, there are not many published data on validation of the assays in patient specimens or clinical diagnosis evaluation in qPCR format. This warrants further investigation on the usefulness of these assays for diagnosis in a clinical setting.

A highly sensitive real-time PCR assay was developed for detection and quantification of *O. tsutsugamushi,* based on the *groEL* gene, which encodes the 60kDa heat shock protein, from contemporary Thai strains. This gene presented as a representative target for molecular diagnostics and had a high degree of conservation across strains. The detection values ranged from 2-31,668 copies/µl when tested using buffy coat specimens [219].

In order to improve molecular diagnostic assays, and to be less time consuming, a SYBR Green-based multiplex real-time PCR targeting 47kDa, citrate synthase (*gltA*) and

ompB genes was established to identify and differentiate STG, TG and SFG rickettsiae, but the clinical diagnostic utility of the assay with patient samples has not been described yet [220]. A *Taq*Man probe-based multiplex real-time PCR assay targeting 47kDa, *groEL,* and human interferon beta (IFN-β gene) genes was developed and evaluated to improve early diagnosis of scrub typhus. The assay had high sensitivity (86.5%, 95% CI 74.2-94.4) and specificity (100%, 95% CI 97.3-100) compared to a four-fold rise in IgM/IgG IFA titre [225]. However, these molecular methods might not be available in most areas where the diseases are endemic.

1.4.4.3.3 Isothermal amplification

A simple, rapid and sensitive method for detection of scrub typhus using loop-isothermal DNA amplification (LAMP) targeting the *groEL* gene has been described with sensitivity comparable with conventional nested-PCR (56kDa gene) [227]. The diagnostic accuracy of the LAMP assay in a clinical setting was evaluated in a prospective study in Thailand. The sensitivity of the LAMP was 53.0% (95% CI 39.0-66.0) and the specificity was 94.0% (95% CI 88.0-98.0). The results showed that diagnostic accuracy was similar to real-time and nested-PCR but better than IgM ICT (47.0% [95% CI 34.0-61.0] sensitivity and 95.0% [95% CI 89.0-98.0] specificity) in early detection. This can be considered as a valid molecular method for early detection of scrub typhus. Results from this study also suggested that combining the LAMP with IgM ICT improved the diagnostic sensitivity (67.0% (95% CI 53.0-79.0) with a specificity of 91.0% (95% CI 83.0-95.0) [228]. Following this finding, an evaluation of the commercial antibody-based ICTs demonstrated overall improved diagnostic performances when used in combination with LAMP showing the potentially utility of them as point-of-care tests [229]. A study from Laos used a LAMP assay targeting *ompB* gene for detection of *R. typhi* and evaluated its clinical performance characteristics. The clinical sensitivity of the assay during development phase (retrospective known clinical specimens) was 48.0% (95% CI 32.5-62.7) and the

specificity was 100% (95% CI 100-100). In a prospective evaluation phase, the specificity remained high with 98.5% (95% CI 97.0-100) but the sensitivity was even lower with 33.0% (95% CI 9.2-56.8). The low sensitivity of the LAMP assay was mainly due to the low *R. typhi* loads in clinical specimens as determined by qPCR (210 copies/ml of blood) [230].

A rapid molecular-based diagnostic test, RPA, using a lateral flow test (RPA-nfo) and real-time fluorescent detection (RPA-exo) has recently been developed targeting the 47kDa gene of *O. tsutsugamushi* or 17kDa gene of *R. typhi* for early diagnosis of acute scrub typhus and murine typhus infections. Similar to LAMP but simpler, the optimal reaction temperature was 37°C, using only one pair of primer for a reaction and the reaction could be completed within 30 minutes. The RPA assay detection level was comparable to that of quantitative PCR method. The assay is simple and rapid with promising high sensitivity and specificity. The results of this development warrants its further evaluation in clinical settings [231].

As described above, many PCR assays have been developed and evaluated in different clinical settings. The analytical accuracy of PCR assays is usually very high. The clinical diagnostic specificity is also generally very high, but the clinical diagnostic sensitivity in endemic settings needs to be improved. The development of PCR assays is still in progress and further evaluation in different clinical settings, especially in the endemic areas, is required.

1.5 Bacteraemia in outpatients

Bacteraemia is the presence of live bacteria in the bloodstream. Like other AUFI, the clinical presentations are non-specific. The most common clinical presentation of bacteraemia is fever. Blood culture is often performed to investigate general causes of bacteraemia. There is little data on community-acquired bacteraemia in febrile patients in

SE Asia [232]. Bacteraemia is relatively uncommon in patients who are not sufficiently unwell to require hospital admission, especially adults [233]. In the USA, the prevalence of bacteraemia could be as low as 1.8% (24/1,350) of non-admitted adult patients [234]. Most literature on bacteraemia in outpatients relates to young children under the age of five years. The prevalence of bacteraemia is generally low and it is rarely found in paediatric outpatients with AUFI, although some may have bacteraemia with *Salmonella* Typhi or *Streptococcus pneumoniae* [235, 236].

1.6 Sub-microscopic malaria

In SE Asia, transmission of malaria is low, unstable and seasonal. Most malaria infections are symptomatic and are detected by malaria smear or RDTs [13, 237]. However, in such low transmission areas (i.e. parasite prevalence by microscopy is <10%), the proportion of sub-microscopic infections is high (70-80%) compared to the areas of high transmission intensity (~20% when parasite prevalence by microscopy is ≥75%) [238].

Thick and thin blood film microscopic examination is the gold standard diagnostic test for detection of malaria parasitaemia, however it requires skilled and experienced technicians to obtain an accurate detection. The limit of detection could be as low as 5-10 parasites/μl of blood in experienced hands, although accuracy is not reliable at a very low parasitaemia <5-10 parasites/μl of blood in routine clinical diagnosis [239]. A systematic review using Bayesian LCMs to analyse the accuracy of malaria diagnostic tests revealed that the sensitivity and specificity of the microscopy in symptomatic patients with *Plasmodium falciparum* ranges between 89.5-96.2% and 97.2-99.7%, respectively in endemic areas [240]. For *Plasmodium* non-falciparum, the sensitivity and specificity ranges between 90.1-98.4% and 95.8-99.8%, respectively [240].

Simple, sensitive and specific antigen-based RDT that detect *P. falciparum* histidine-rich protein II (HRP-II), pan-malaria or species specific (Plasmodium) lactate dehydrogenase (pLDH), or aldolase antigens are widely used [13]. The limit of detection is around 100 parasites/µl of blood [240]. These RDT formats are useful in clinical settings where microscopy is not available or there is a lack of a trained microscopist. pLDH and aldolase antigen are rapidly cleared after effective treatment, but HRP-II antigen may persist for longer than a month [239]. This limits the use of HRP-II-based RDT in high transmission areas [13]. The HRP-II-based RDTs are as good as the routine clinical microscopy for *P. falciparum* in endemic areas, but microscopy remains more reliable for detection of non-falciparum malaria [239, 240].

Sub-microscopic malaria infection is identified using PCR technique. PCR is not used in routine diagnosis of malaria infection. It is used for research purposes such as epidemiology studies of malaria in low transmission settings. PCR has been developed for malaria detection based on several target genes such as 18S *rRNA* [241-245], *tRNA* [246], and mitochondrial genes [247]. Of these 18S *rRNA* is most commonly used. Finger-prick capillary blood specimens (≤5µl), blood spot (~30µl), or small volume blood specimens (100-200µl) have been normally used for PCR assays [245-248]. The sensitivity of the PCR assays is limited by the specimen volume. A qPCR assay targeting 18S *rRNA* gene was developed and assessed using large volume blood specimen (1ml). The assay was able to detect malarial DNA as low as 22 parasites/ml of blood (95% CI 21.8-74.9) [249]. This assay is currently used to detect *Plasmodium* species and quantify their parasitaemia for active screening of asymptomatic subjects in malaria elimination strategies.

1.7 Study rationale and objectives

1.7.1 Rationale for the study

AUFI is a common clinical presentation in tropical countries and infections presenting this way still remain a major public health problem, especially in rural areas which lack good infrastructure, clinical laboratory facilities and have poor sanitary conditions [1]. Many common causes of febrile illness in the tropics have no specific symptoms and signs, making diagnosis in the clinic difficult [11]. It is rarely possible to establish a definitive diagnosis clinically in patients with AUFI and they are difficult to diagnose in clinic based laboratories that often have limited resources, and thus, under-reported. Accurate and rapid diagnostic tests are usually not available in disease endemic areas. Therefore, very few clinical diagnoses of these infections are confirmed by laboratory investigation. However, dengue, leptospirosis and rickettsial infection (especially scrub typhus and murine typhus) are likely to be common causes of AUFI among the population living along the Thailand-Myanmar border where this study took place and have been shown, in clinical studies, to be common in rural SE Asia generally [6, 7, 11].

Dengue is the most widespread mosquito-borne viral disease. It is estimated by WHO that 50-100 million dengue infections occur annually worldwide, with a 30-fold increase incidence over the past five decades [35]. Increasing incidence and spread of dengue fever have been reported in nine countries within the SE Asia region between 1985-2005 and has continued to increase in recent years [43]. In addition, the most recent estimate of global risk shows that there are around 390 million dengue infections (96 million symptomatic) globally per year, which is over three times higher than the figure estimated by the WHO [38].

Rickettsial infections are the second most frequently reported non-malaria causes of fever in SE Asia [250] and are a significant risk among refugee populations, especially those living along the Thailand-Myanmar border [171, 251], Scrub typhus and murine typhus are the most common rickettsial causes of AUFI and are widely endemic in Asia, with an estimated one million cases of scrub typhus occurring each year [167, 172].

The incidence of leptospirosis is estimated to be approximately 10-100 per 100,000 in the humid tropics. The disease incidence may be more than 100 per 100,000 during outbreaks and in high-exposure risk groups [108].

The gold standard tests for these infections rely on paired serology which is time consuming and results in delayed diagnosis confirmation. Field deployment of accurate rapid tests would facilitate the timely diagnosis of these diseases [2, 15].

While malaria has decreased globally, non-malaria causes of fever have become apparent and remain a major cause of AUFI in SE Asia [250]. This is in line with the data reported by the six clinics of the Shoklo Malaria Research Unit (SMRU) located along the Thailand-Myanmar border. While malaria cases have been decreasing steadily, non-malaria fever cases of unknown diagnosis have remained consistently high over the period October 2007 to September 2014 (Figure 1.22 and Figure 1.23).

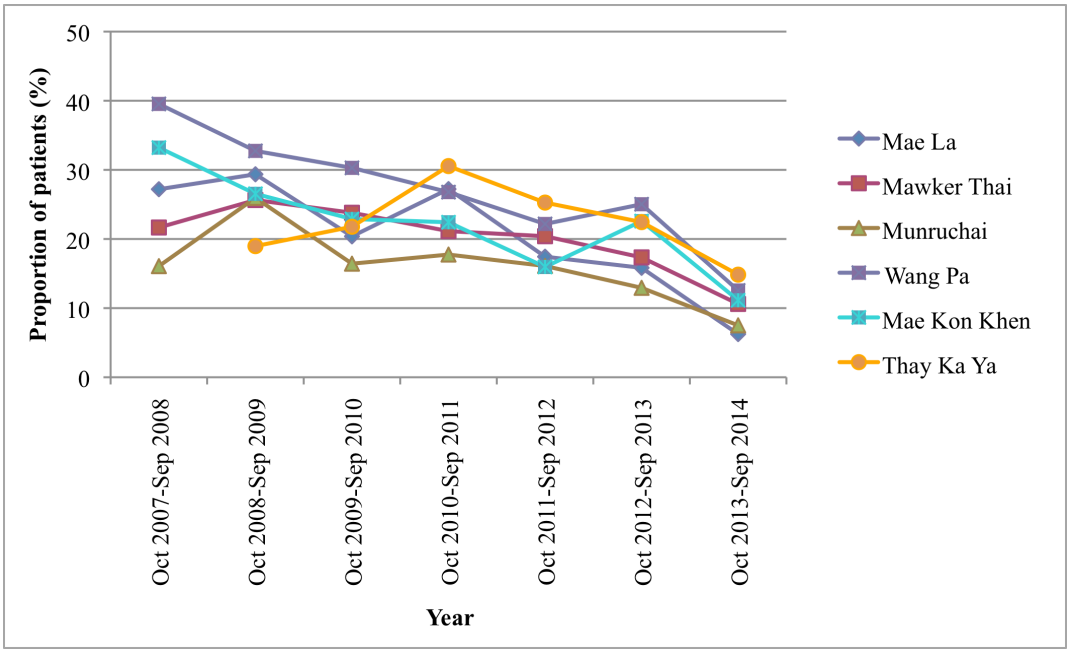


Figure 1.22 Proportion of febrile patients with malaria presenting to SMRU clinics between October 2007 and September 2014.

Note: Data for Oct 2007-Sep 2008 from Thay Ka Ya was not available.

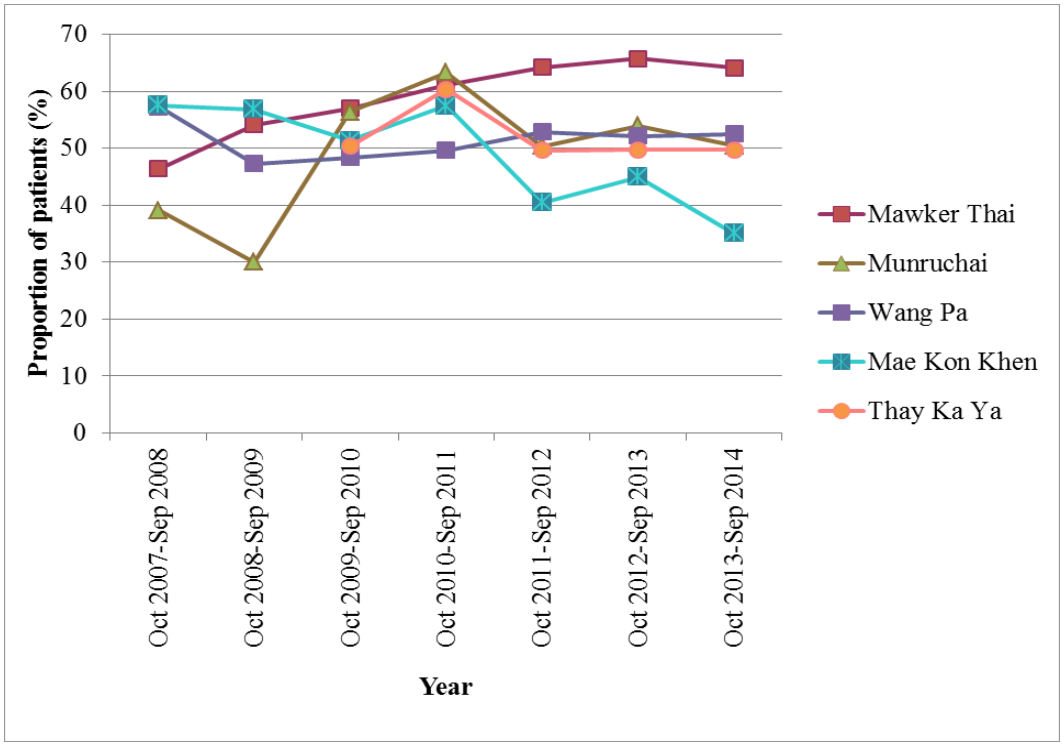


Figure 1.23 Proportion of febrile patients with unknown diagnosis presenting to SMRU clinics between October 2007 and September 2014.

Note: Data from Mae La, and for Oct 2007-Sep 2009 from Thay Ka Ya were not available.

This present study represented a relatively new avenue of investigation for SMRU, and built on the study of causes of fever in pregnancy conducted between 2004-2006. This work found that malaria was the most common cause of fever (55.5%, 227/409). However, dengue, leptospirosis, scrub typhus and murine typhus were also common in a sub-cohort of detailed fever investigations and follow-up (47.3%, 96/203). In addition, co-infections were reported in 3.9% (8/203) of cases, of which malaria and rickettsia co-infection was the majority [10]. Improved field-based diagnostic tools are urgently required for dengue, leptospirosis and rickettsial infections, especially in non-malaria febrile patients. A recent prospective study evaluating tests for more rapid diagnosis of dengue in the SMRU clinic population found that a combination of rRT-PCR or NS1 Antigen detection plus IgM antibody detection was required for accurate confirmation of infection [16].

Improved understanding of the incidence of these infections in the SMRU clinic population would assist the development of treatment algorithms and patient management for fever cases, which would have relevance for the wider rural tropical population.

1.7.2 Research question

What are the optimal testing strategies for diagnosis of the common causes of AUFI in a rural SE Asia clinic?

1.7.3 Hypotheses to be tested

The main hypothesis of this study is that current diagnostic tests, usually based on paired serology, for common non-malaria causes of fever (dengue, leptospirosis, and rickettsial infections) in SE Asia are inadequate for clinical use and lead to under-diagnosis of these infections. The diagnostic accuracy and the clinical value of using rapid tests needs to be determined to diagnose causes of fever in a rural SE Asian clinical setting. The plan was to answer the following questions:

1. What are the causes of AUFI in patients presenting with fever where a definitive diagnosis cannot be given in the field (excluding malaria)?

2. How can we best diagnose these diseases in the field with a clinically useful turnaround time?

    a. Can new generation rapid tests for dengue and rickettsial infections be used to reliably determine the cause of infection in patients with AUFI in rural SE Asia?

    b. Can new molecular diagnostic assays replace current serological methods as gold standard tests for confirmation of these infections?

1.7.4 Aims and objectives

The principal objective of the study is to determine optimal testing strategies for dengue, leptospirosis, and rickettsial infections (scrub typhus and murine typhus).

Additional objectives were to:

1. Determine the diagnostic accuracy and clinical value of using new generation rapid diagnostic tests and molecular diagnostic assays to diagnose causes of fever in a rural SE Asian clinical setting.

2. Assess inter-operator variation for the interpretation of the ICT results.

3. Evaluate the molecular diagnostic assays as alternatives to the ICT, and as possible replacement for the current serological methods as gold standard test for the confirmation of acute dengue and scrub typhus infections.

4. Estimate the occurrence of common causes of AUFI, including dengue, leptospirosis, rickettsial infections (focus on scrub typhus and murine typhus), and other invasive bacterial infections in the SMRU clinic population.

5. Improve the understanding of these infections in the population to assist the development of the treatment algorithms and patient management of non-malaria fever cases.

6. Assess the impact of dual infection with malaria:

    a. Quantify and detect sub-microscopic malaria infections in febrile patients

    b. Assess the impact of malaria infection on the performance characteristics of the diagnostic tests under evaluation.

2 Materials and Methods

2.1 Study sites

2.1.1 Location

Shoklo Malaria Research Unit (SMRU) is a field station of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand and is part of the Mahidol-Oxford Tropical Medicine Research Unit (MORU). SMRU is located in Mae Sot, approximately 500km from Bangkok in Tak province in the Northwest Thailand, and runs five main clinics on the Thailand-Myanmar (Burma) border (Figure 2.1). It has been providing medical support and conducting research in this area since 1986 (http://www.shoklo-unit.com/).

Wang Pha, approximately 30km north of Mae Sot, and Mawker Thai, approximately 60km south of Mae Sot, are two of the largest clinics which provide healthcare for the migrant population from Myanmar living and working along the border.

Mae La temporary shelter (Mae La) is the largest camp for refugees from Myanmar, housed in an area of 4km^2. It is located in hills adjoining the Myanmar border, approximately 70km north of Mae Sot.

Patients were recruited from SMRU migrant clinics at Wang Pha and Mawker Thai and from the clinic at Mae La refugee camp.

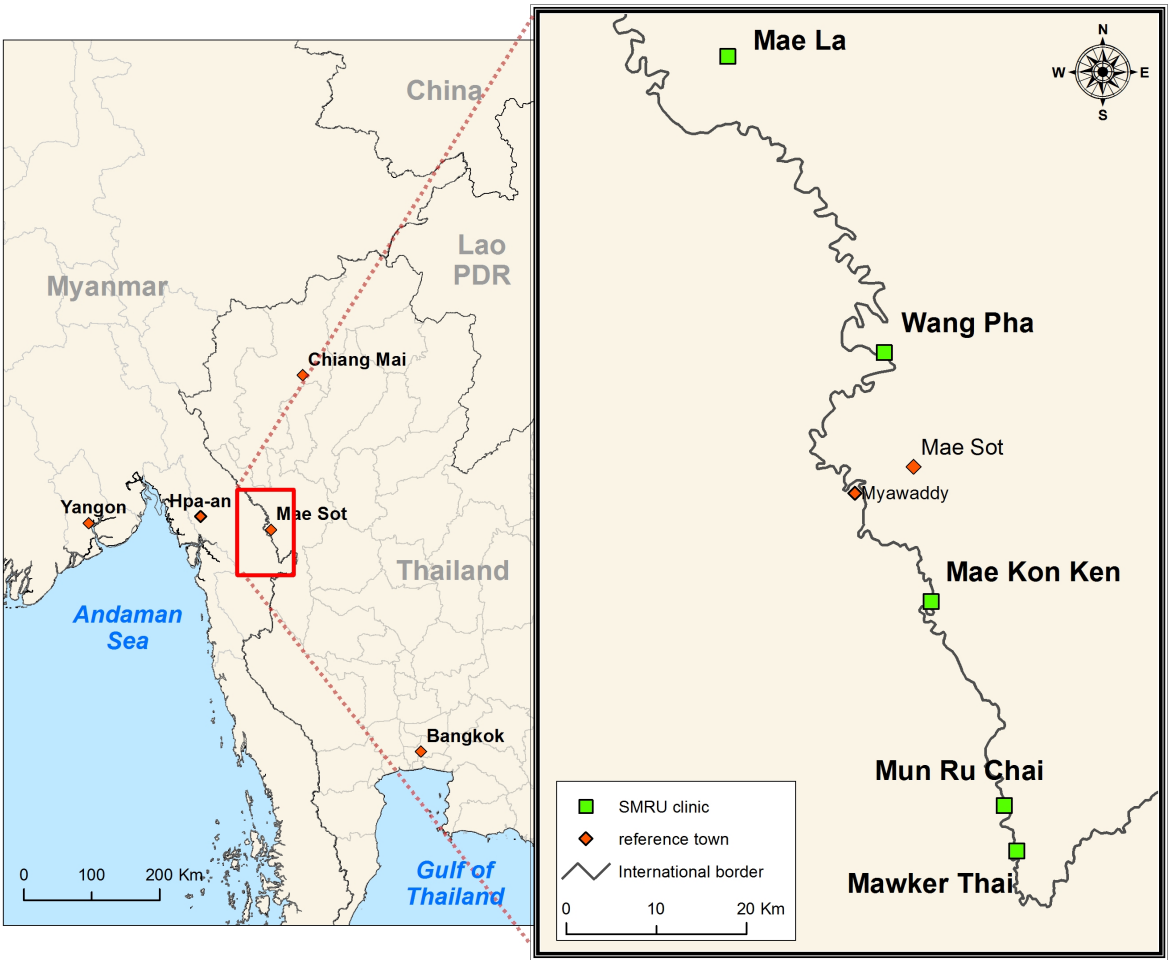


Figure 2.1 Map of SMRU clinics (Mae La, Wang Pha, Mawker Thai, Mun Ru Chai, and Mae Kon Ken).

Note: Thay Ka Ya is an extension of Wang Pha clinic and is not included on the map.

Map courtesy of Daniel Parker.

2.1.2 Population

Migrants

The true number of the migrant population living along the Thailand-Myanmar border is unclear. In 2011, it was estimated that there were 154,000-190,000 migrants, both registered and unregistered, living in Tak province and 30,000-45,000 villagers in Myanmar living within the SMRU catchment areas [252]. The estimated size of the

migrant population served by the SMRU clinics was 46,175-97,010 migrants between October 2007 and September 2014 (http://www.shoklo-unit.com/).

Refugees

Refugees from Myanmar have been arriving in Thailand since 1984. Mae La is the largest of the nine refugee camps along the Thailand-Myanmar border. In February 2011, the registered refugee population in Mae La camp was 25,093 people, accounting for 32.5% of all registered Myanmar refugees in Thailand. The majority of the Myanmar refugee population in Thailand are from the Karen ethnic group (>75%) (figure from http://www.tbbc.org). The number of registered refugees is thought to be a large underestimation of the true camp population, which has been recently estimated at >42,000.

2.2 Study design

The work contained in this thesis was divided into two parts and both parts were conducted in parallel (Figure 2.2). This prospective fever diagnostic study (FDS) was run in three outpatient clinics (OPD) at Mae La, Wang Pha, and Mawker Thai, recruiting patients aged at least five years with undifferentiated febrile illness. Patients less than five years old were not included due to blood volume requirements of the study.

PART I. (Non-malaria)

Patients presenting to the clinics with non-malaria fever (see section 2.4) were recruited into the main part, PART I, of the study (Figure 2.2). Prospective evaluations of various new diagnostic methodologies were performed to determine the aetiology of common non-malaria infections, describe the clinical features of patients presenting with these infections, relate their clinical status to the test performance, and also set up a bio-bank of well-characterised clinical specimens for future diagnostic test evaluations. The study sites were in known malaria endemic areas, and recent research has showed that

asymptomatic/sub-microscopic malaria infection, detected by an ultra-sensitive qPCR assay, acts as a *Plasmodium* spp. reservoir [249, 253]. The ultra-sensitive qPCR assay was performed in the present study to detect sub-microscopic malaria infection. The impact of sub-microscopic malaria infection on the performance of the non-malaria diagnostic tests under evaluation was investigated.

PART II. (Malaria)

Patients presenting with fever and malaria according to microscopy or rapid diagnostic test (RDT) were recruited into PART II of the study (Figure 2.2). The non-malaria diagnostic tests under evaluation were performed in patients with malaria as well as in non-malaria to detect dual infections. There were insufficient patients in this group to evaluate the impact of microscopic/RDT confirmed malaria infection on the performance of the non-malaria tests.

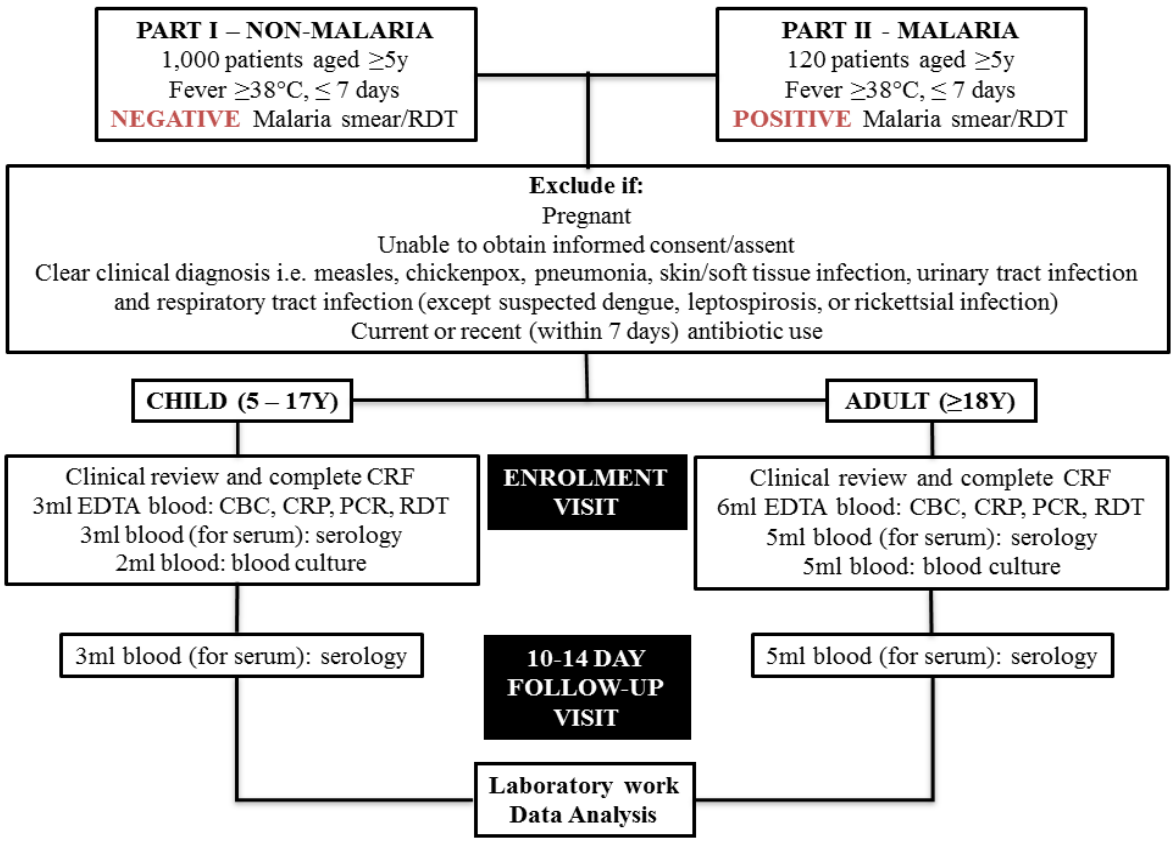


Figure 2.2 Study flow chart.

2.3 Study size

PART I.

Patients with non-malaria undifferentiated fever were recruited at all sites and recruitment numbers were closely monitored to ensure that the recruitment was even throughout the year. Three local data sets were used to estimate an appropriate sample size. The studies of Ellis *et al.* [11], Suttinont *et al*. [7], and the SMRU pilot data (Watthanaworawit *et al*. [254]) are summarised below (Table 2.1).

Table 2.1 Summary of the three fever studies using similar methodology and conducted in Thailand.

Patient numbers	**Ellis *et al.***	**Suttinont *et al.***	**Watthanaworawit *et al.***
Total patients [n]	613 adults	845 adults	162 adults
Dengue [n (%)]	9 (1.5%)	64 (7.6%)	72 (44.4%)
Leptospirosis [n (%)]	107 (17.5%)	312 (36.9%)	6 (3.7%)
Rickettsial infection [n (%)]	36 (5.9%)	206 (24.4%)	16 (9.9%)

From these data, it was estimated that we might diagnose the following numbers of cases in 1,000 participants:

1. Dengue: 15-440 cases
2. Leptospirosis: 40-370 cases
3. Rickettsial infection: 60-240 cases

The diagnostic accuracy of the tests was estimated with the following precision (95% confidence intervals) based on the number of true positive (sensitivity) or true negative (specificity) cases as defined by the reference test result [255] (Table 2.2). For example, 100 confirmed cases (true positive) as defined by the reference test would have to

be recruited, if the sensitivity of the new test was estimated to be 90% with the 95% confidence interval of ±5.9%. If the prevalence of disease in the study population was 10%, then there would be 10 confirmed cases per 100 patients seen at the clinic. Therefore, 1,000 patients would need to be recruited to obtain 100 confirmed cases.

Table 2.2 Estimated diagnostic accuracy of the tests [255].

Number of cases required*	**Estimated test sensitivity or specificity (95% CI)***		
	50%	**70%**	**90%**
50	± 13.9%	± 12.7%	± 8.3%
100	± 9.8%	± 9.0%	± 5.9%
200	± 6.9%	± 6.4%	± 4.2%
300	± 5.7%	± 5.2%	± 3.4%
400	± 4.9%	± 4.5%	± 2.9%

*As defined by the reference test result.

PART II.

Data on the expected frequency of dual infections is scarce: Ellis and colleagues reported dengue, leptospirosis, or rickettsial co-infections in 17% (26/155) of malaria cases [11]. Therefore, it was estimated that in order to detect 20 (95% CI 11-25) patients with dual infection, 120 patients with microscopically or rapid diagnostic test confirmed malaria (*Plasmodium falciparum, Plasmodium vivax,* or mixed infection) should be recruited. Every week, the first eligible malaria patient was recruited at each of the three study sites, resulting in recruitment of 120 patients over approximately 40 weeks. This pilot data will be used to inform the design of a larger study on dual infections.

2.4 Patient recruitment

Inclusion criteria

For PART I, any patient aged at least five years presenting to the clinic with a fever of at least 38°C of up to seven days duration and negative for malaria according to malaria smear or RDT were eligible. For PART II, patients aged at least five years presenting with the above criteria but with microscopically or RDT confirmed malaria infection were eligible (Figure 2.2).

Exclusion criteria

Pregnancy, currently taking antimicrobials or had taken antimicrobials in the last seven days prior to presentation, or unable to give informed consent (assent for paediatric patients) were exclusion criteria for both parts. A positive malaria smear or RDT and patients with a clear clinical diagnosis (except clinically suspected dengue, leptospirosis, rickettsial infection, or typhoid), for example; measles, chickenpox, pneumonia [256], skin/soft tissue infection (e.g. cellulitis), urinary tract infection and respiratory tract infection were excluded from PART I. Patients with a clear secondary clinical diagnosis as above were also excluded from PART II (Figure 2.2).

2.5 Malaria screening tests

Microscopy (smear) and/or RDT were performed for malaria diagnosis at SMRU outpatient clinics as part of routine malaria screening. Microscopic examination was performed by trained microscopists. At MLA clinic, only the smear was performed. For WPA and MKT clinics, the smear was performed on a quality control (QC) week (one week per month, also called "smear week"). The RDT was routinely performed on a non-smear week by trained clinical staff. Additional smears were performed to confirm malaria infections when requested by a clinician/medic.

2.5.1 Microscopy (smear)

According to the SMRU standard operating procedure (SOP, T2), thick and thin films were prepared on the same slide per specimen. A blood smear one centimetre in diameter containing two to three drops from finger prick was used for the thick film and one drop for the thin film. The slide was then placed onto a homemade hot plate to dry. Then, the blood films were fixed using methanol (BDH Prolabo VWR, Fontenay-sous-Bois, France). After that, the slide was stained using 10% Giemsa staining solution (BDH Prolabo VWR, Fontenay-sous-Bois, France) for 20 minutes. Finally, the slide was washed with clean water and placed on a rack to dry. The slide was examined under a microscope (Olympus, Tokyo, Japan) at a magnification of x1000. The results were recorded as negative or positive with *Plasmodium* species, stages and parasitaemia. Trophozoites were counted on the thick film against 500 white blood cells if there were <500/500 WBC or on the thin film if there were ≥500/500 WBC. On the thin film, the trophozoites were counted against 1,000 red blood cells (RBC). Schizonts and gametocytes were counted on the thick film against 500 WBC.

2.5.2 Immunochromatographic (ICT) rapid diagnostic test (RDT)

SD BIOLINE Malaria Antigen P.f/Pan POCT (Standard diagnostics, Inc., Gyeonggi-do, Republic of Korea) was used in the clinics. The test was designed to detect malaria infection in human blood and to differentiate between *P. falciparum* infection through detection of HRP-II antigen and infection from other *Plasmodium* species (Pan) through detection of pLDH antigen. Briefly, 5µl of capillary blood from a finger prick was added into the round sample well, followed by all of the assay diluent from the diluent ampoule into the square well of test device. The test was incubated at room temperature for a minimum of 15 minutes (up to 30 minutes) and results were interpreted no later than 30 minutes. The test has three lines: “C” (control), “Pan” (*Plasmodium* species; *P. vivax, P. malariae* and *P. ovale*), and “P.f” (*P. falciparum*) lines. An unclear background and/or an

absence of the control line indicated an invalid result. According to the SMRU SOP (RT5), a positive "Pan" line was recorded as positive for *P. vivax*. Although this test is reported to be accurate, like any diagnostic test false results may occur; for example if the test is not performed correctly or due to interfering substances. Rheumatoid factor-contained in specimens and infection with *Schistosoma mekongi* have been shown to cause false positive results. It should also be noted that HRP-II antigen may persist for longer than a month following acute infection, limiting usage in high transmission areas [257].

2.6 Clinical data and specimen collection

Patients were sampled at the acute febrile illness visit and at a follow-up visit (day 10-14). A case report form (CRF, see Appendix 1) was completed by a clinician or medic trained to provide medical assistance at SMRU clinics, to record clinical symptoms and signs relating to the illness.

The acute specimens were collected at the time of initial clinical assessment. For adult patients (≥18 years old), a total of 16ml of blood was collected. This included 6ml venous blood in a sterile Ethylenediaminetetraacetic acid (EDTA) tube (BD Vacutainer; Becton Dickinson, Franklin Lakes, NJ, USA) for CBC, CRP level, pathogen specific PCRs, and RDTs; 5ml venous blood (for serum) in a sterile plain tube (Teklab, Country Durham, UK) for serology; and 5ml of blood (BacT/ALERT FA bottle, BioMérieux, Durham, NC, USA) for bacterial culture. The blood was taken prior to antibiotic treatment. For paediatric patients (5-17 years old), a total of 8ml of blood was collected. This included 3ml venous blood in a sterile EDTA tube (BD Vacutainer) for CBC, CRP, pathogen specific PCRs and RDTs; 3ml venous blood (for serum) in sterile plain tube (Teklab) for serology; and 2ml of blood (BacT/ALERT PF bottle) for bacterial culture taken prior to antibiotic treatment (Figure 2.2).

Patients were reviewed at a study-specific 10-14 day follow-up visit and a repeat 5ml (patients aged ≥18 years) or 3ml (patients aged 5-17 years old) venous blood specimen was collected into a sterile plain tube for serology.

Specimens were transported to the SMRU Mae Sot laboratories on a daily basis in a cool box for EDTA blood and serum, or ambient temperature for blood culture.

After clinical review, the patient was treated according to the suspected clinical diagnosis following standard clinical algorithms in operation in SMRU clinics. Additionally, patients with malaria (PART II) were treated according to the SMRU malaria guideline (http://www.shoklo-unit.com/resources). Severely unwell patients were admitted to the clinic inpatient department for treatment.

2.7 Specimen processing and storage

Specimens were allocated a specimen number on arrival at SMRU Mae Sot laboratories. A unique ID was given to each specimen aliquot and entered into databases (SMRU Microbiology database for blood culture and SMRU Haematology database for EDTA blood (CBC and CRP), both Microsoft Access (Microsoft, Redmond, WA, USA). Freezerworks sample management software was used for EDTA blood and serum specimen storage (Dataworks Development, Mountlake, WA, USA).

Aliquot storage

Prior to separation all EDTA blood and serum specimens were stored in a refrigerator or cool box. The blood specimens from adults and children were divided as shown in Figure 2.3 and Figure 2.4. All aliquots (whole blood, plasma, buffy coat, packed red cell and serum) were stored at -80°C until further processing. The aliquot of plasma for dengue rapid diagnostic test was tested immediately or was stored in a refrigerator prior to testing, no later than three days following collection.

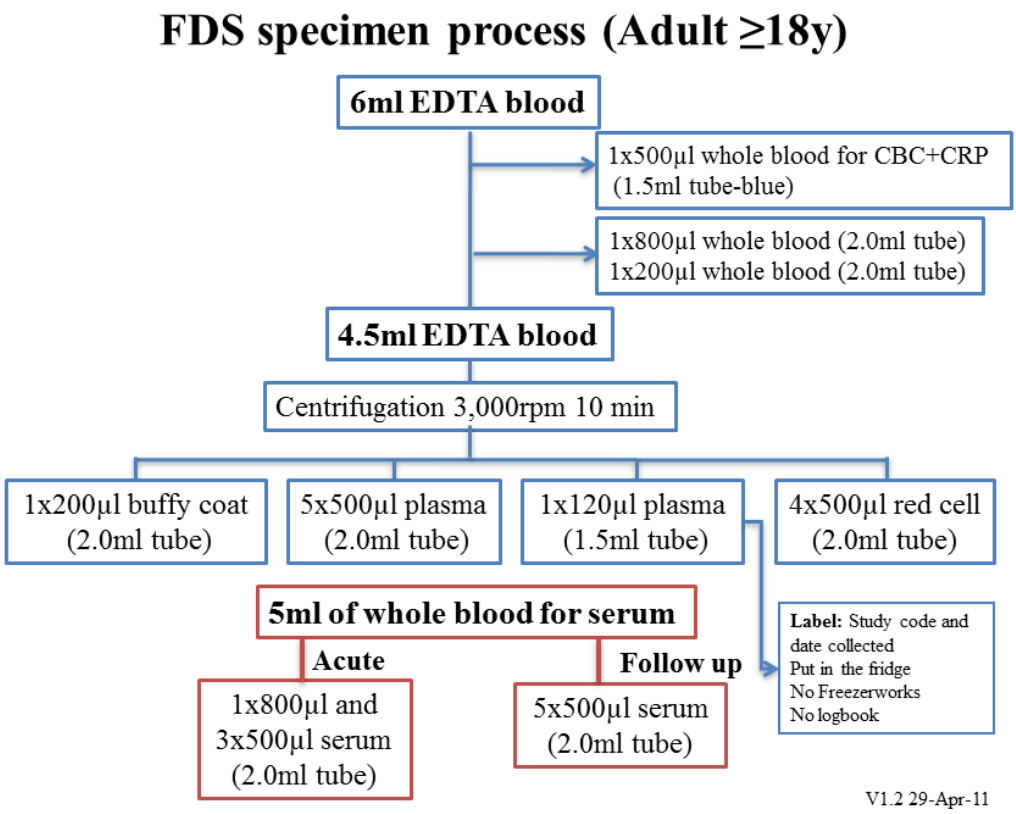


Figure 2.3 EDTA blood and serum specimen processing for adult patients.

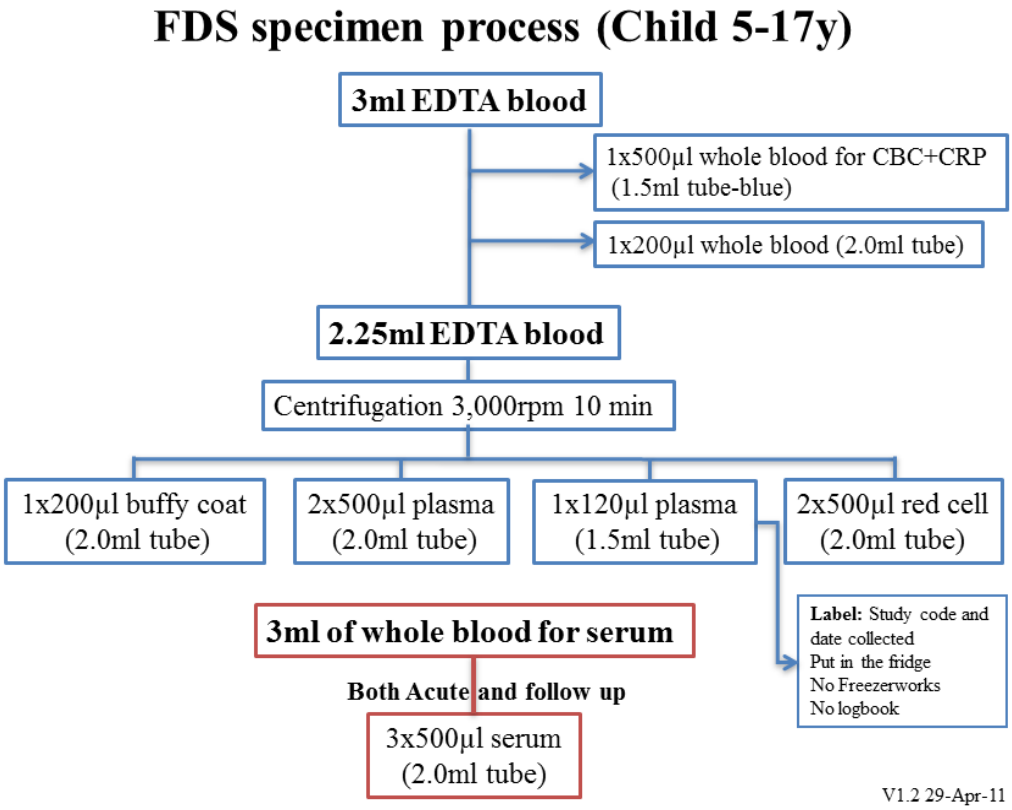


Figure 2.4 EDTA blood and serum specimen processing for child patients.

2.8 Ethical considerations

Written informed consent/assent was obtained from all patients, or their legal guardian if aged less than 18 years, prior to enrolment in the study following the scheme outlined in Table 2.3. Consent was obtained by the medic, nurse or doctor assessing the patient at the initial clinic visit. The study consent/assent and patient information sheet were translated into Karen and Burmese languages. For patients who were unable to read or write, the forms were read out and explained by a doctor, medic or nurse who spoke the same language as the patient or his/her guardian. A thumbprint was used to confirm and a witness signed the consent form. The study was reviewed by the Tak Province Community Ethics Advisory Board (TCAB-01-11), and was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand (MUTM 2011-008-01) and Oxford Tropical Research Ethics Committee, University of Oxford, Oxford, UK (OXTREC 42-10).

Table 2.3 Informed consent and assent form scheme.

Age	Informed Consent Form	Assent Form
Less than 7 years	Parent signs ICF for permission	-
7 to 12 years	Parent signs ICF for permission	Child signs Assent Form
13 to 17 years	Parent and child sign the same ICF	-
18 years and over (Adult)	Adult signs ICF	-

2.9 Laboratory methods

All specimens were processed in SMRU Mae Sot laboratories and/or sent to the relevant reference laboratories [i.e. MORU and Armed Forces Research Institute of Medical Science (AFRIMS)] for specialist serological assays. SMRU SOPs were followed for processing of all specimens.

2.9.1 Dengue

2.9.1.1 Enzyme Immuno Assay (EIA) or Enzyme-Linked Immunosorbent Assay (ELISA)

Paired serum specimens were sent to AFRIMS for ELISA testing for detection of antibodies to dengue and JE, IgM/IgG antibodies [70, 258]. The interpretation algorithm of dengue virus and JE virus infection using in-house AFRIMS ELISA is shown in Appendix 2. An acute dengue infection was defined as the presence of anti-dengue IgM of 40 EIA units or more in an acute specimen with the anti-dengue IgM unit being greater than the anti-JE IgM unit. Acute primary and secondary dengue infections were defined when the ratio of anti-dengue IgM to anti-dengue IgG were more than or equal to 1.8 or less than 1.8, respectively. If the anti-dengue IgM was less than 40 units in acute specimens, an increase in anti-dengue IgG between acute- and convalescent-phase specimens to an absolute value of more than 100 units was used to define acute secondary dengue infection. If there were rising anti-dengue or anti-JE IgG of more than 100 units, but the anti-dengue IgM units were less than the anti-JE IgM units in the convalescent specimen, then acute secondary flavivirus infection was defined. Specimens were considered negative for acute infection if specific antibodies were absent in paired sera specimens collected at least 5-7 days apart as defined in Appendix 2. The EIA units were calculated from binding index (BI) multiplied by 100. The BI was calculated from average OD of specimen minus OD of negative control divided by OD of weak positive control minus OD of negative control.

2.9.1.2 Immunochromatographic (ICT) rapid diagnostic test (RDT)

Acute plasma specimens were prospectively tested as part of routine clinical microbiology laboratory processes at SMRU, using the SD BIOLINE Dengue Duo RDT (Standard diagnostics, Inc.). The test was designed to detect both NS1 antigen and IgM/IgG antibodies to dengue virus in human serum, plasma or whole blood. Briefly, three drops (~100µl) of plasma were added into the sample well marked "S" for the NS1 antigen test. For the IgM/IgG antibody test, 10µl of plasma were added into the sample well marked "S", followed by four drops of diluent into the assay diluent well. The test was incubated at room temperature and the results were interpreted at 15 minutes. The NS1 antigen test has two lines (control line "C" and test line "T") whereas the IgM/IgG antibody test has three lines (control line "C", IgM line "M", and IgG line "G"). An absence of control line "C" indicates an invalid result. The test was read by three independent readers who were on duty in the microbiology laboratory. The readers did not confer while reading the test and the results were blinded (each reader did not see the results of the other readers). Subsequently, the consensus result (i.e. 2/3 readers with the same interpretation) was used for final interpretation.

2.9.1.3 Dengue group specific one step SYBR Green based real-time RT-PCR (rRT-PCR) assay

Nucleic acid extraction

Acute plasma specimens were processed in batches. Nucleic acid was extracted from plasma specimens using an automated nucleic acid extractor, MagCore HF16 (RBC Bioscience, New Taipei City, Taiwan) with MagCore Viral Nucleic Acid Extraction Kits, cartridge code 202 (RBC Bioscience). In summary, 10µl of carrier RNA (1mg/ml) and 20µl of Proteinase K (10mg/ml) were added into 1.5ml sample tubes (provided by kit), followed by 200µl of plasma. The tubes were then placed into the MagCore/T-rack, and

the cartridge code 202 programme was run on the MagCore HF16, with a 60µl elution volume selected. Extracts were processed immediately or stored at 2-8°C prior to testing within 24 hours or at -80°C for longer storage.

rRT-PCR amplification

A one-step SYBR Green based rRT-PCR assay (Dengue group specific) targeting the core protein gene was performed using the original primer sequences from Shu *et al.* [259], and the modification from Watthanaworawit *et al.* [16]. The assay was performed using the SuperScript III Platinum SYBR Green One-Step qRT-PCR systems (Invitrogen, Carlsbad, CA, USA). Amplification was carried out using the ABI 7500 FAST real-time PCR system (Applied Biosystems, Foster City, CA, USA). The primer set was designed for universal detection of dengue viruses. It does not provide the specific serotype (DENV-1 to 4) of the virus [259] (Table 2.4).

Briefly, each 25µl reaction mix contained 10µl of extracted RNA, 1µl of each primer (10µM each) (Sigma-Aldrich, Saint Louis, MO, USA), 12.5µl of 2X SYBR Green reaction mix (containing 0.4mM of each deoxynucleotide triphosphate (dNTP) and 6mM magnesium sulfate ($MgSO_4$)) and 0.5µl of SuperScript III RT/Platinum *Taq* mix (including RNaseOUT ribonuclease inhibitor). The reaction mix and *Taq* were supplied in the SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen). The amplification conditions consisted of reverse transcription at 50°C for 30 minutes, *Taq* inhibitor inactivation at 95°C for 5 minutes, followed by 45 cycles of 95°C for 10 seconds, 54°C for 30 seconds and 72°C for 30 seconds, and hold at 95°C for 1 minute. Melting curve analysis was used to confirm the specific amplicons, starting from 55°C to 95°C with 1%/step increment (continuous detection).

An internal control, a human ribonuclease P (*RNaseP*) rRT-PCR, was used to detect the presence of inhibitors in the specimens [260]. The primer and probe sequences

are shown in Table 2.4. Each 25µl reaction mix comprised 5µl of extract, 0.5µl of each primer and probe (10µM each) (Sigma-Aldrich), 12.5µl of 2X reaction mix containing 0.4mM of each dNTP and 6mM $MgSO_4$ (supplied with the SuperScript III Platinum One-Step qRT-PCR kit, Invitrogen), 0.5µl of SuperScript III RT/Platinum *Taq* mix (supplied with the kit, Invitrogen) and 5.5µl of nuclease-free water, using the same amplification conditions as dengue group specific rRT-PCR assay as described above. No template control (NTC), negative extraction control (NEG) and positive control were included in each PCR run.

Table 2.4 Primer and probe sequences for Dengue group specific and *RNaseP* rRT-PCR.

Name	Sequences (5'➔3')
DN_F [259]	CAATATGCTGAAACGCGNGAGAAA
DN_R [259]	CCCCATCTNNYCANNATYCCTGCTGT
*RNaseP*_F [260]	AGATTTGGACCTGCGAGCG
*RNaseP*_R [260]	GAGCGGCTGTCTCCACAAGT
*RNaseP*_P [260]	FAM-TTCTGACCTGAAGGCTCTGCGCG-BHQ1

F=forward primer, P=probe, and R=reverse primer; BHQ1=Black Hole Quencher1 as the quencher, and FAM=6-carboxy-fluorescein as the fluorescent reporter dye

Interpretation

Dengue group specific assay

The positive control as well as unknown positive specimens should cross the threshold (14,000) with a cycle threshold (Ct) value lower than the Ct value of negative controls and unknown negative specimens. The Ct value of the positive control was around 20 (5µl/reaction was applied and equivalent to 16.7 plaque forming unit (PFU)/reaction of

DENV1-3 and 166.7 PFU/reaction of DENV-4). Specimens were considered positive when the melting temperature was between 80.0°C and 83.3°C. All specimens melting below, or having no associated peak to these temperatures were considered negative. There was always some fluorescent signal for negative controls and negative specimens. This was either non-specific or primer-dimer-related. The fluorescence of these specimens usually started to decrease before they reached the plateau and their melting temperature was below 80.0°C (usually around 70°C). If unknown specimens had a Ct value similar to NTC or NEG but their fluorescence curve reached the plateau, melting curves were checked for a peak around 80.0°C and 83.3°C, in which case they were positive. In cases of doubt, the PCR product of the specimen was separated by agarose gel electrophoresis to check for a 170 base pairs (bp) band corresponding to dengue virus.

Dengue virus control strains were obtained from AFRIMS, consisting of DENV-1 (Hawaii strain), DENV-2 (New Guinea C strain), DENV-3 (H87 strain), and DENV-4 (814669 strain). A DENV1-4 mixture was used for positive control. Nuclease-free water and plasma extract from a healthy donor were used as NTC and NEG, respectively.

RNaseP (internal control) assay

All clinical specimens should exhibit *RNaseP* reaction curves that cross the threshold (20,000) line at or before a Ct value of 35, indicating the presence of the human *RNaseP* gene. Failure to detect *RNaseP* in any of the clinical specimens indicated improper extraction of nucleic acid from clinical materials resulting in loss of nucleic acid or carry-over of PCR inhibitor from clinical specimens, improper assay setup and execution, or reagent or equipment malfunction. If a specimen was *RNaseP* negative, then nucleic acid extraction and PCR were repeated. If all of the specimens were *RNaseP* negative (including positive control), PCR failure was suspected and the assay was repeated. Plasma extract from a healthy donor, nuclease-free water and its extract were used as positive

control, NTC and NEG, respectively. A false positive result in NTC or NEG indicated contamination in the process. If NTC was positive, the PCR run was repeated. If NEG was positive, the extraction and PCR run were repeated.

2.9.1.4 Nested-RT PCR for dengue virus serotyping assay

All RNA extracts that were positive by the dengue group specific one step SYBR Green based rRT-PCR assay (section 2.9.1.3) underwent nested-RT PCR for dengue virus serotyping [80], modified by AFRIMS, Bangkok, Thailand [74]. The AFRIMS protocol was introduced and optimised at the SMRU laboratory for this study. Amplification was performed using a GeneAmp PCR System 9700 (Applied Biosystems). The 50µl reaction mixture for the RT-PCR step contained 5µl of extracted RNA, 0.25µl of AmpliTaq DNA polymerase (5 Unit [U]/µl) (Gene Systems, Rockville, MD, USA), 5µl of 10X PCR buffer II (supplied with AmpliTaq DNA polymerase, Gene Systems), 3µl of 25mM magnesium chloride ($MgCl_2$) (supplied with AmpliTaq DNA polymerase, Gene Systems), 1µl of GeneAmp 10mM dNTPs mixed with deoxythymidine triphosphate (dTTP) (Applied Biosystems), 1.25µl of each primer (D1, and D2; 10 pico moles (pmol)/µl each, Table 2.5) (Sigma-Aldrich), 0.25µl of 1M dithiothreitol (DTT) (Sigma-Aldrich), 0.1µl of Avian Myeloblastosis Virus reverse transcriptase (AMV-RT) (10U/µl) (Promega, Madison, WI, USA), and 32.9µl of nuclease-free water. The amplification conditions consisted of reverse transcription at 42°C for 60 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 1 minute and 72°C for 2 minutes, then hold at 4-10°C. The amplified RT-PCR product was diluted to 1:50 dilution to use in the nested-PCR step. The 50µl reaction mixture of the nested-PCR step contained 5µl of 1:50 diluted RT-PCR product, 5µl of 10X PCR buffer II (supplied with AmpliTaq DNA polymerase, Gene Systems), 5µl of 25mM $MgCl_2$ (supplied with AmpliTaq DNA polymerase, Gene Systems), 1µl of GeneAmp 10mM dNTPs mixed with dTTP (Applied Biosystems), 1.25µl of each primer (D1, TS1, TS2, TS3, and TS4; 10pmol/µl each, Table 2.5) (Sigma-Aldrich), 0.25µl of AmpliTaq DNA

polymerase (5U/µl) (Gene Systems), and 27.5µl of nuclease-free water. The amplification conditions consisted of 25 cycles of 94°C for 30 seconds, 53°C for 1 minute, 72°C for 2 minutes, and then hold at 4-10°C. Positive and negative controls were included in each PCR run. PCR products were analysed using 1.8% (w/v) agarose gel electrophoresis, run at 100 volts for 1 hour and 45 minutes. The products were visualised and image acquisition was captured by a molecular imager Gel Doc XR+ System (BioRad, Hercules, CA, USA) together with the Quantity One 1-D analysis software (BioRad).

Interpretation

A positive RT-PCR result was identified by the detection of a DNA band of 511bp. The result was considered negative when no band of 511bp was observed. A specimen containing DENV-1, 2, 3, 4 was identified by the detection of a DNA band of 482, 119, 290, or 392bp, respectively. Nuclease-free water and the same set of positive controls from dengue group specific one step SYBR Green based rRT-PCR assay (section 2.9.1.3) were used as negative and positive controls, respectively. Assays were valid only if the positive and negative controls had appropriate results.

Table 2.5 Primer sequences for dengue virus serotyping nested-RT PCR assay.

Name*	Sequences (5'➔3')
D1	TCAATATGCTGAAACGCGCGAGAAACCG
D2	TTGCACCAACAGTCAATGTCTTCAGGTTC
TS1	CGTCTCAGTGATCCGGGGG
TS2	CGCCACAAGGGCCATGAACAG
TS3	TAACATCATCATGAGACAGAGC
TS4	CTCTGTTGTCTTAAACAAGAGA

*Primer sequences from Lanciotti *et al.* [80].

2.9.2 Leptospirosis

2.9.2.1 16S *rRNA* (*Leptospira* spp.) quantitative real-time PCR (qPCR) assay

Nucleic acid extraction

Extraction was performed using the MagCore HF16 (RBC Bioscience) as described above (section 2.9.1.3). Bacterial DNA was extracted from acute plasma specimens in batches, using the MagCore Genomic DNA Whole Blood Kit, cartridge code 102 (RBC Bioscience). Briefly, 20µl of Proteinase K (10mg/ml) were added into 1.5ml sample tubes (provided by kit), followed by 200µl of plasma. The tubes were then placed into the machine, and the cartridge code 102 programme was run, with a 100µl elution volume selected. Extracts were processed immediately or stored at 2-8°C prior to testing within 24 hours or at -80°C for longer storage.

qPCR amplification

The 16S *rRNA* qPCR assay was performed using Platinum *Taq* DNA polymerase kit (Invitrogen) and the amplification was carried out using the ABI 7500 FAST real-time PCR system (Applied Biosystems). The primers and probe were based on Smythe *et al.* and Slack *et al.* [147, 150] (Table 2.6). The assay was modified from Thaipadungpanit *et al.* [149]. A total reaction mixture of 20µl included 5µl of extracted DNA, 2µl of 10X buffer (supplied with *Taq* DNA polymerase, Invitrogen), 0.5µl of forward primer (10µM) (Sigma-Aldrich), 1µl of reverse primer (10µM) (Sigma-Aldrich), 0.2µl of probe (5µM) (Sigma-Aldrich), 1µl of dNTPs (5mM) (Roche, Basel, Switzerland), 1.7µl of $MgCl_2$ (50mM) (supplied with *Taq* DNA polymerase, Invitrogen), 0.25µl of *Taq* DNA polymerase (0.5U/reaction) (Invitrogen), and 8.35µl of nuclease-free water. A 10-fold serial dilution of linearised plasmid DNA from *L. interrogans* starting from 10,000 to 0.1 copies/µl (six points) was used in duplicates for standard control. Nuclease-free water and plasma extract from a healthy donor were used for NTC and NEG, respectively.

Amplification was performed in the fast mode of the ABI 7500 FAST real-time PCR system (Applied Biosystems), consisting of 98°C for 20 seconds, followed by 45 cycles of 95°C for 3 seconds and 58°C for 30 seconds. The human *RNaseP* PCR was used as an internal control (Table 2.4) [260]. The PCR reaction mix (both concentration and volume) and amplification condition were exactly the same as those of the 16S *rRNA* qPCR as described above. Plasma extract from healthy donor, nuclease-free water and its extract were used as positive control, NTC and NEG, respectively and they were included in each PCR run.

Table 2.6 Primer and probe sequences for 16S *rRNA* (*Leptospira* spp.) qPCR assay.

Name	Sequences (5'➔3')
Lepto_F [147]	CCCGCGTCCGATTAG
Lepto_R [147]	TCCATTGTGGCCGRACAC
Lepto_P [147, 150]	FAM-CTCACCAAGGCGACGATCGGTAGC-BHQ1

F=forward primer, P=probe, and R=reverse primer; BHQ1=Black Hole Quencher1 as the quencher, and FAM=6-carboxy-fluorescein as the fluorescent reporter dye

Interpretation

16S rRNA (Leptospira spp.) assay

All specimens were considered positive when they had amplification curves above a fixed threshold (10,000) and a Ct value of ≤40. The standard control at 1 copy/µl was expected to give a Ct value of 36 and the standard control of 1-10,000 copies/µl should have amplified in duplicate (if not, the run was repeated). Non-amplification of the 0.1 copies/µl control was deemed acceptable [149]. An accurate quantification was determined

using the control data points between 1-10,000 copies/µl (five points). Runs were valid only if the standard and negative controls had appropriate results.

RNaseP (internal control) assay

Amplification curves that cross a fixed threshold line at 10,000 should be obtained from all clinical specimens. A PCR run was valid when the positive control had an expected Ct value of 35 and no Ct value for the negative controls. Any failure to obtain an appropriate result resulted in an action being taken as described above (section 2.9.1.3).

16S rRNA (L. interrogans) plasmid DNA preparation

Insertion of fresh PCR product into vector

Plasmid DNA control was prepared using pCR8/GW/TOPO TA cloning kit (Invitrogen). Amplified PCR product of the 16S *rRNA* gene (*L. interrogans*) was inserted into the TOPO vector. A total volume of 6µl of the cloning reaction consisted of 3µl of fresh PCR product, 1µl of salt solution, 1µl of water and 1µl of the TOPO vector. The reaction was mixed gently and was incubated for five minutes at room temperature. Then, the reaction tube was placed on ice for two minutes to stop ligation. After this step, the cloning reaction was used for the transformation step.

Transformation

The inserted cloning vector was used to transform competent *Escherichia coli* (supplied with the cloning kit, Invitrogen) using a chemical transformation technique. A 2µl volume of the inserted cloning vector from the previous step was added into a vial of competent *E. coli.* The reaction was mixed gently and was incubated on ice for 10 minutes. After that, the cells were heat-shocked for 30 seconds at 42°C without shaking. The tube was immediately transferred to ice for 2-3 minutes. Then, 250µl of room temperature

S.O.C. medium (supplied with the cloning kit, Invitrogen) was added into the transformation tube. The tube was capped tightly and was shaken horizontally (200rpm) at 37°C for one hour in the Thermo-Shaker TS-100C (Biosan, Riga, Latvia). After this step, transformants were checked by streaking 10µl and 20µl of the transformation reaction onto prewarmed Luria-Bertani (LB) selective agar plates containing 100µg/ml spectinomycin. The plates were incubated over night at 37°C in an aerobic incubator.

Analysing transformants

Several hundred colonies should be produced on the LB selective plate for an efficient cloning reaction. The transformation efficiency should be $\geq 10^5$ colony forming unit (CFU)/µg. The 16S *rRNA* (*Leptospira spp.*) qPCR was performed to confirm the presence and correct orientation of the insert, using a single colony on the LB selective plate. The colony was added directly to the qPCR reaction. Once confirmation of the insert was obtained, stocks of transformant *E. coli* were prepared. A single colony was inoculated onto a LB selective plate and incubated at 37°C overnight. The clone was checked by the 16S *rRNA* qPCR again before storage. The transformant *E. coli* was transferred to skim-milk tryptone glucose glycerol (STGG) storage medium and stored at -80°C for long term storage. Another single colony was inoculated onto a LB selective plate and incubated at 37°C overnight for plasmid extraction. Circular plasmid DNA was extracted using the QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany), following the manufacturer's instructions.

Linearisation of plasmid DNA

The circular plasmid DNA was linearised using *Eco*RI restriction enzyme. A total volume of 100µl of restriction reaction consisted of 10µl of 10X React3 buffer, 5µl of *Eco*RI (10U/µl), 50µl of circular plasmid DNA and 35µl of water. The reaction was incubated at 37°C for two hours, followed by the heat-inactivation step at 80°C for 20

minutes. After this step, the quantity of linearised plasmid DNA was measured using the Qubit Fluorometer (Invitrogen). The linearised plasmid DNA was stored at -80°C for long term storage.

2.9.3 Rickettsial infections

2.9.3.1 Enzyme-linked Immunosorbent Assay (ELISA)

All rickettsial work on acute and convalescent serum specimens was performed at the Serology laboratory, Microbiology Department, MORU, Bangkok. Specimens were screened by ELISA for detection of scrub typhus [207, 261] and murine typhus IgM antibodies [262]. U-bottom 96 well microtitre plates (Sterilin, Newport, UK) were coated with 100µl/well of *O. tsutsugamushi* antigen (1:1,000 dilution for Karp and Kato strains, and 1:1,500 dilution for Gilliam strain) or *R. typhi* antigen (1:3,000 dilution) in phosphate-buffered saline (PBS) (Oxoid, Hampshire, UK). 100µl/well of PBS with no antigen were added for blank plates. The plates were covered with a plastic wrap and stored at 4°C for two days. After this time, the coated plates were washed three times with wash buffer (0.1% Tween 20 in PBS), then the plates were blocked with 200µl/well of blocking buffer (5% skim milk in wash buffer) for one hour, and finally rinsed with wash buffer three times. All specimens were tested in duplicate. Serum specimens were diluted 1:100 in blocking buffer, 100µl/well, in microtitre plates with and without antigen (blank) and incubated for one hour at room temperature. The plates were washed four times with wash buffer, and then 100µl/well of anti-human IgM horseradish peroxidase (HRP) conjugate (1:10,000 dilution) (Invitrogen) was added before incubation for one hour at room temperature. Following this, the plates underwent four washes with wash buffer and then 100µl/well of tetramethylbenzidine (TMB) substrate (Invitrogen) was added and the plates incubated for 10 minutes at room temperature in the dark. After 10 minutes, 100µl/well of stop solution (1M HCl) (Merck, Kenilworth, NJ, USA) was added to the plates. The plates

were then read at a wavelength of 450nm (subtracted a reference OD value read at 630nm) using a Multiskan EX microplate reader (ThermoFisher Scientific, Waltham, MA, USA). Positive and negative controls were included in each plate. Serum specimens that were positive by IgM IFA (≥1:25,600 titre) for scrub typhus or murine typhus were used as positive controls for scrub typhus or murine typhus assay, respectively. The ODs from the wells without antigen were used to subtract background absorbance. All patient specimens that had an OD of at least a 1.4-fold increment or a high static OD between acute and convalescent specimens, or were positive by PCR (described in section 2.9.3.4) on the acute specimen, were selected for further IFA testing.

2.9.3.2 Indirect Immunofluorescence Assay (IFA)

The IFA assay was used to determine IgM antibody titre against *O. tsutsugamushi* antigen (Karp, Kato, and Gilliam strains) and *R. typhi* antigen (Wilmington strain) [208, 263]. This assay was performed on all specimens that were suggestive of scrub typhus or murine typhus infection by ELISA and PCR results as described in section 2.9.3.1 and 2.9.3.4. Slides for the scrub typhus and murine typhus IFA assays were obtained from the Australian Rickettsial Reference Laboratory (ARRL; Geelong, Victoria, Australia). Serum specimens were serially diluted two-fold from 1:100 to 1:25,600 in PBS (Oxoid) buffer containing 2% (w/v) skim milk powder (Becton Dickinson). Paired acute and convalescent serum specimens from the same patient were performed on the same slide side-by-side. Positive and negative controls were included for each patient. 2µl of diluted sera was dropped on each well of the slide, incubated at 37°C for 30 minutes in a humid chamber, and washed four times (five minutes each time) in PBS. Following this, the slide was dried and then 2µl of diluted fluorescein isothiocyanate (FITC) anti-human IgM conjugate [anti-human IgM FITC conjugate (Invitrogen) diluted in 2% PBS-skim milk powder diluent containing 0.005% (w/v) Evans blue counterstain] was added on to each well of the slide, incubated at 37°C for 30 minutes in a humid chamber, and washed four times (five minutes

each time) in PBS. Fluorescence mounting medium (Dako, Glostrup, Denmark) was dropped onto the slide and a cover slip added. Slides were examined by epifluorescence microscopy (BX60; Olympus, Tokyo, Japan) at a magnification of x400 by two readers. The binding endpoint titre was determined as the highest titre that showed fluorescence. If the discrepancy in titre between two readers was greater than two-fold, a third reader result was obtained. The highest titre from the two readers with closest agreement was then used as the binding endpoint titre.

Patients were considered to have evidence of acute infection when a ≥four-fold increase in titre or seroconversion between acute and convalescent specimens or a static titre of ≥1:25,600 was observed.

2.9.3.3 Immunochromatographic (ICT) rapid diagnostic test (RDT)

Acute plasma specimens were tested using SD BIOLINE Scrub typhus IgM ICT test for the detection of IgM antibody against *O. tsutsugamushi* (Boryong strain) [229]. The test was obtained from Standard diagnostics, Inc., Kyonggi-do, Korea and is currently not commercially available. Briefly, 10µl of plasma was added into the sample well, followed by three drops of assay diluent, and then the test was incubated at room temperature and the result was interpreted as positive or negative at 15 minutes. The test has two lines, a control line "C" and a test line "T". An absence of "C" line indicates an invalid result. The tests were read by three independent readers who were on duty in the routine clinical microbiology laboratory at SMRU. The readers did not confer while reading the test and the results were blinded (each reader did not see the results of the other readers). Subsequently, the consensus result (i.e. 2/3 readers with the same interpretation) was used for final interpretation.

2.9.3.4 Quantitative real-time PCR (qPCR)

The extraction method and qPCR assays for detection of rickettsia were modified from the original papers by Paris and Castonguay-Vanier and described in the Lao Oxford Mahosot Wellcome Trust Research Unit (LOMWRU) 2010 SOPs [210, 217, 218, 223]. The assays were then introduced and optimised at SMRU laboratory as described below (section 2.9.3.4.1 - 2.9.3.4.3).

Nucleic acid extraction

Buffy coats from acute blood specimens were analysed in batches. Bacterial DNA was extracted from buffy coat using the QiaAmp DNA Blood Mini kit (Qiagen) following the manufacturer's instructions [264]. The lysis incubation time at 56°C was increased from 10 minutes to one hour and elution volume was decreased to 100µl to obtain a higher DNA concentration. Briefly, 20µl of protease was added into 1.5ml microcentrifuge tube (Axygen, Corning, NY, USA), followed by 200µl of buffy coat and 200µl of buffer AL, then the mixture was vortexed for 15 seconds using vortex-genie 2 (Scientific Industries, Bohemia, NY, USA). The tube was incubated at 56°C for one hour. Following this, the tube was briefly centrifuged using Biofuge PICO (Heraeus, Hanau, Germany) to remove any drops inside of the tube lid, and then 200µl of absolute ethanol (VWR, Arlington Heights, IL, USA) was added and mixed by vortexing for 15 seconds. The mixture from the previous step was transferred to a spin column, and was centrifuged at 8,000rpm for one minute. After that, the column was placed into a new collection tube and the filtrate was discarded in 1% Virkon (DuPont, Suffolk, UK) container for disinfection. 500µl of buffer AW1 was added into the column and the column was centrifuged at 8,000rpm for one minute. The column was placed into a new collection tube and the filtrate was discarded. After this step, 500µl of buffer AW2 was added into the spin column and the column was centrifuged at full speed (13,000rpm) for three minutes. The filtrate was

discarded and the spin column was put back into the collection tube, and was centrifuged at full speed for one minute. Following this, the spin column was placed into a new 1.5ml microcentrifuge tube (Axygen) and 50µl of buffer AE was added into the spin column. The column was centrifuged at 8,000rpm for one minute after incubation for five minutes at room temperature. Then, the step of adding 50µl of buffer AE into the spin column, incubation for five minutes and centrifugation at 8,000rpm for one minute were repeated. The eluted DNA was then mixed by pipetting 10 times and then was split in two tubes of equal volume. Extracts were processed immediately or stored at 2-8°C prior to testing within 24 hours or at -80°C for longer storage.

2.9.3.4.1 47kDa qPCR assay (*O. tsutsugamushi*-specific)

The 47kDa assay was performed using the Platinum Quantitative PCR SuperMix-UDG kit (Invitrogen), and the amplification was carried out using the ABI 7500 FAST real-time PCR system (Applied Biosystems). This assay was used to detect the presence of *O. tsutsugamushi* DNA. Primers and probe were based on Jiang *et al.* [210] (Table 2.7). The sequences were based on the 47kDa outer membrane protein gene of the Karp, Kato, Gilliam, Boryong and TH1817 strains of *O. tsutsugamushi*. Each 25µl of reaction mix contained 1µl of extracted DNA, 15µl of Platinum Quantitative PCR SuperMix-UDG (Invitrogen), 0.25µl of each primer (OtsuFP630 and OtsuRP747; 10µM each) (Sigma-Aldrich), 0.5µl of probe (OtsuPR665; 10µM) (Sigma-Aldrich), and 8µl of Nuclease-free water. A 10-fold serial dilution of linearised plasmid DNA (47kDa gene from *O. tsutsugamushi* strain UT76) from 1 to 1,000 copies/µl (four points) was used in duplicate for standard controls and nuclease-free water was used for negative control. The amplification condition comprised 50°C for 2 minutes, 95°C for 2 minutes, followed by 45 cycles of 95°C for 15 seconds, and 60°C for 30 seconds. Standard and negative controls were included in each PCR run. The human *RNaseP* PCR was used as an internal control (only tested on the first 327 buffy coat specimens; primer and probe sequences are shown

in Table 2.4), using the same amplification condition as 47kDa assay as above, but the concentration of the PCR mix was slightly different: each 25µl of reaction mix contained 1µl of extracted DNA, 15µl of Platinum Quantitative PCR SuperMix-UDG (Invitrogen), 0.25µl of each primer (40µM each) (Sigma-Aldrich), 0.5µl of probe (10µM) (Sigma-Aldrich), and 8µl of nuclease-free water. Nuclease-free water and buffy coat extract from a healthy donor were used as negative and positive controls.

Interpretation

47kDa qPCR assay

The standard controls were added in duplicates (1, 10, 100, 1,000 copies/µl). The standard at 10, 100, and 1,000 copies/µl had to be amplified for at least one replicate for each dilution. If not, the run was repeated. The 1 copy/µl standard might not be amplified and this was acceptable as 3-10 copies/µl could be detected in the original paper [210]. There had to be no amplification in the negative control. All specimens were considered positive when they had amplification above the fixed threshold (25,000). The quantification was calculated from 10-1,000 copies/µl of standard controls (three points). For any specimens that were positive at less than 10 copies/µl, quantification was not reported. All positive specimens were repeated and analysed for an accurate quantification using the data point between 10-100,000 copies/µl of standard controls (five points). The standard control of 10-100,000 copies/µl had to be amplified in duplicate (if not, the run was repeated). Any specimens that were positive less than 10 copies/µl were considered positive, but lower than the level of accurate quantification. Hence, the quantity was not reported.

RNaseP (internal control) assay

Amplification curves that cross a fixed threshold line at 50,000 should be obtained from all clinical specimens. A PCR run was valid when the positive control had an expected Ct value of 23 and no Ct value for the negative controls. Any failure to obtain an appropriate result resulted in action being taken as described above (section 2.9.1.3).

This internal control assay was performed only on the first 327 specimens and only once for each specimen when testing a set of rickettsial PCRs including 47kDa, 17kDa and *ompB* assays, since the same DNA extracts were used for these assays and all of the 327 specimens showed very consistent positive Ct values.

Table 2.7 Primer and probe sequences for 47kDa, 17kDa and *ompB* qPCR assays.

Name	Sequences (5'➔3')
OtsuFP630 [210]	AACTGATTTTATTCAAACTAATGCTGCT
OtsuRP747 [210]	TATGCCTGAGTAAGATACRTGAATRGAATT
OtsuPR665 [210]	FAM-TGGGTAGCTTTGGTGGACCGATGTTTAATCT-TAMRA
R17K128F2 [217, 223]	GGGCGGTATGAAYAAACAAG
R17K238R [217, 223]	CCTACACCTACTCCVACAAG
R17K202TaqP [217, 223]	FAM-CCGAATTGAGAACCAAGTAATGC-TAMRA
Rt557F [218]	TGGTATTACTGCTCAACAAGCT
Rt678R [218]	CAGTAAAGTCTATTGATCCTACACC
Rt640BP [218]	FAM-CGCGATCGTTAATAGCAGCACCAGCATTATCGCG-BHQ1

BHQ1=Black Hole Quencher1 as the quencher, FAM=6-carboxy-fluorescein as the fluorescent reporter dye, and TAMRA=6-carboxytetramethylrhodamine as the quencher

2.9.3.4.2 17kDa qPCR assay (*Rickettsia*-specific)

The 17kDa qPCR assay was performed using the Platinum Quantitative PCR SuperMix-UDG kit (Invitrogen) and amplification was carried out using the ABI 7500 FAST real-time PCR system (Applied Biosystems). The 17kDa assay was used to detect bacterial species from the *Rickettsia* genus. Primer and probe sequences were based on Wright *et al.,* and Jiang *et al.* [217, 223] (Table 2.7). Each 25µl of reaction mix contained 1µl of extracted DNA, 15µl of Platinum Quantitative PCR SuperMix-UDG (Invitrogen), 1µl of each primer (R17K128F2 and R17K238R; 10µM each) (Sigma-Aldrich) and probe (R17K202TaqP; 10µM) (Sigma-Aldrich), and 6µl of nuclease-free water. A 10-fold serial dilution of linearised plasmid DNA (17kDa gene from *R.typhi* strain Wilmington) from 1 to 1,000 copies/µl (four points) was used in duplicates for standard controls and nuclease-free water was used as negative control. The amplification conditions consisted of 50°C for 2 minutes, 95°C for 2 minutes, followed by 45 cycles of 95°C for 15 seconds, and 60°C for 30 seconds (same as 47kDa assay). Standard and negative controls were included in each PCR run.

Interpretation

The interpretation was the same as 47kDa assay described above (section 2.9.3.4.1) with a fixed threshold at 30,000 for 17kDa assay. All positive specimens from this assay were tested further by a specific *R. typhi ompB* assay.

2.9.3.4.3 *ompB* (*R. typhi*) qPCR assay

All positive specimens by 17kDa qPCR assay were assessed with the *ompB* qPCR assay. This assay was used to detect the unique sequence in the outer membrane protein B gene (*ompB*) of *R. typhi.* The primer and probe sequences were based on Henry *et al.* [218]. The reporter and quencher dyes of the probe were changed from Tetrachlorofluorescein (TET) and 4-(dimethylaminoazo) benzene-4-carboxylic acid

(DABCYL) to 6-carboxy-fluorescein (FAM) and Black Hole Quencher1 (BHQ1), respectively (Table 2.7). This assay was performed using the Platinum Quantitative PCR SuperMix-UDG kit (Invitrogen) and the amplification was carried out using the ABI 7500 FAST real-time PCR system (Applied Biosystems). Each 25µl of reaction mix contained 1µl of extracted DNA, 15µl of Platinum Quantitative PCR SuperMix-UDG (Invitrogen), 1µl of each primer (Rt557F and Rt678R; 10µM each) (Sigma-Aldrich) and probe (Rt640BP; 10µM) (Sigma-Aldrich), and 6µl of nuclease-free water. A 10-fold serial dilution of linearised plasmid DNA (*ompB* gene from *R.typhi* strain Wilmington) from 1 to 1,000 copies/µl (four points) was used in duplicates for standard controls and nuclease-free water was used for negative control. The amplification conditions consisted of 50°C for 2 minutes, 94°C for 2 minutes, followed by 45 cycles of 94°C for 15 seconds, and 60°C for 30 seconds. Standard and negative controls were included in each PCR run.

Interpretation

The interpretation was the same as 47kDa assay described above (section 2.9.3.4.1) with a fixed threshold at 30,000 for *ompB* assay. The 1 copy/µl standard might not be amplified: Henry *et al.* could detect 3 copies/µl or more [218].

47kDa, 17kDa and ompB plasmid DNA preparation

Circular plasmid DNA controls were obtained from MORU, consisting of 47kDa gene (*O. tsutsugamushi* strain UT76), 17kDa gene (*R.typhi* strain Wilmington) and *ompB* gene (*R.typhi* strain Wilmington) inserted in pGEM-T Easy vector (Promega, Madison, WI, USA). Linearisation of plasmid DNA was performed in the molecular suite at SMRU laboratory, using appropriate restriction enzymes. The 47kDa plasmid was linearised using *Sal*I-HF. The 17kDa and *ompB* plasmids were linearised using *Spe*I-HF. A total volume of 30µl of linearised reaction for 47kDa plasmid contained 3µl of 10X NEBuffer 4, 2µl of *Sal*I-HF, 4µl of circular plasmid DNA (250-300ng/µl), and 21µl of distilled water. For the

17kDa and *ompB* plasmids, a total volume of 30µl of linearised reaction contained 3µl of 10X NEBuffer 4, 3µl of 10X BSA, 2µl of *Spe*I-HF, 4µl of circular plasmid DNA (250-300ng/µl), and 18µl of distilled water. The reactions were then incubated at 37°C for two hours, followed by the heat inactivation step at 80°C for 20 minutes. After this step, 2µl of each linearised plasmid DNA was checked for the complete cut by performing a 1% agarose gel electrophoresis. Then, the linearised plasmids were purified using QIAquick PCR purification kit (Qiagen), followed the manufacturer's instructions. The quantity of lineared plasmid DNA was measured using the Qubit Fluorometer (Invitrogen). The linearised plasmid DNA was stored at -80°C for long term storage.

2.9.4 Bacterial infection

2.9.4.1 Blood culture

The SMRU microbiology laboratory routinely participates in the Thailand National External Quality Assurance Scheme (Thailand NEQAS) in clinical microbiology from the Department of Medical Science (DMSc); unit code MI 1637. All blood culture specimens were processed using BacT/Alert FA culture bottles or BacT/Alert PF culture bottles with the BacT/ALERT Microbial Detection System (BioMérieux) for qualitative detection of aerobic and facultative anaerobic microorganisms. This was performed through the routine process of the SMRU microbiology laboratory following the Blood Culture SOP (MBL-2-B). Blood culture bottles were incubated for five days before being declared negative. If a positive growth signal was detected, a Gram stain was performed and the relevant method for bacterial identification was followed, using standardised SOPs. The results were sent to physicians for patient management.

2.9.5 Malaria

The malaria molecular assays used in this study were modified from the original papers [265-267] by the malaria molecular laboratory at MORU, Bangkok, Thailand. The assays were introduced and optimised at the SMRU laboratory for this study.

2.9.5.1 18S *rRNA* (*Plasmodium* spp.) qPCR assay

Nucleic acid extraction

Plasmodium spp. DNA was extracted from 200µl of packed red blood cells (RBC), from acute blood specimens, in batches. The QiaAmp DNA blood mini kit (Qiagen) was used, following the manufacturer's instructions. For the final elution step, DNA was eluted with 100µl of nuclease-free water (two times) and incubated for five minutes, then dehydrated using vacuum concentrator plus (Eppendorf, Hamburg, Germany) and re-suspended with 10µl of AE buffer. Extracts were processed immediately or stored at 2-8°C prior to testing within 24 hours or at -20°C for longer storage.

qPCR amplification

The 18S *rRNA* qPCR assay was used to detect the 18S *rRNA* gene of the *Plasmodium* genus. Primers and probe were based on Kamau *et al.* [265] (Table 2.8). The assay was performed using the QuantiTect Multiplex PCR No ROX kit (Qiagen) and amplification was carried out using the RotorGene 6000 (Corbett Research, Mortlake, Australia). Each 10µl of reaction mix contained 2µl of extracted DNA, 5µl of 2X QuantiTect Multiplex PCR No ROX master mix (Qiagen), 0.4µl of primer mix (10µM) (Sigma-Aldrich), 0.2µl of probe (10µM) (Sigma-Aldrich), and 2.4µl of nuclease-free water. Amplification consisted of 95°C for 15 minutes, followed by 50 cycles of 94°C for 15 seconds and 60°C for 1 minute. A 5-fold serial dilution from 0.128-2,000 parasite/µl (seven points) was used for standard controls in duplicates (only one replicate for the 2,000 parasites/µl). Positive and negative controls were included in each PCR run.

Interpretation

All specimens were considered positive when they had amplification above a fixed threshold (0.03) and a Ct value of ≤38. The standard control at 0.64 parasites/µl was expected to give a Ct value of 35. The standard control of 0.64-400 parasites/µl had to be amplified in duplicate (if not, the run was repeated). An accurate quantification was determined using the data points between 0.64-400 parasites/µl (five points). All positive specimens by this assay were subjected to *P. falciparum* and *P. vivax* speciation by microsatellite nested-PCR.

Table 2.8 Primer and probe sequences for 18S *rRNA* (*Plasmodium* spp.) qPCR assay.

Name*	**Sequences (5'➔3')**
Forward primer	GCTCTTTCTTGATTTCTTGGATG
Reverse primer	AGCAGGTTAAGATCTCGTTCG
Probe	FAM-ATGGCCGTTTTTAGTTCGTG-TAMRA

*Primer and probe sequences from Kamau *et al.* [265].

2.9.5.2 Microsatellite nested-PCR for *P. falciparum* and *P. vivax* speciation

The microsatellite nested-PCR assay was performed on all specimens that were positive for the 18S *rRNA* qPCR assay. The primer sequences were based on Anderson *et al.* and Imwong *et al.* [266, 267]. The PfPK2 and 3.502 primer sets were used for *P. falciparum* and *P. vivax*, respectively (Table 2.9). The primers mix PfPK2 F/PfPK2 3R, and PfPK2 F/PfPK2 R were used for the first round and second round PCR for *P. falciparum,* respectively and the primers mix PV 3.502 F1/PV 3.502 R1, and PV 3.502 F2/PV 3.502 R1 were used for the first round and second round PCR for *P. vivax,* respectively.

Table 2.9 Primer sequences for *P. falciparum* and *P. vivax* microsatellite nested-PCR speciation.

Name	Sequences (5'➔3')
PfPK2 F [267]	CTTTCATCGATACTACGA
PfPK2 3R [267]	CCTCAGACTGAAATGCAT
PfPK2 R [267]	AAAGAAGGAACAAGCAGA
PV 3.502 F1 [266]	CCATGGACAACGGGTTAG
PV 3.502 R1 [266]	TCCTACTCAGGGGGAATACT
PV 3.502 F2 [266]	GTGGACCGATGGACCTAT

The assays were performed using MyTaq DNA polymerases (Bioline, London, UK) and amplification was carried out using GeneAmp PCR System 9700 (Applied Biosystems). The same reaction mixture was used for the first round and second round PCR for both *P. falciparum and P. vivax*. Each 16µl of reaction mixture contained 2µl of extracted DNA, 1.5µl of 5X MyTaq reaction buffer (supplied with MyTaq DNA polymerase, Bioline), 0.75µl of 50mM $MgCl_2$ (supplied with MyTaq DNA polymerase, Bioline), 0.375µl of 5mM dNTPs (Roche), 1.5µl of 2.5µM primers mix (Sigma-Aldrich), 0.06µl of MyTaq DNA polymerase (5U/µl) (Bioline), and 9.815µl of nuclease-free water.

The amplification conditions for each PCR were different. For *P. falciparum,* the first round PCR consisted of 30 cycles of 94°C for 1 minute, 52°C for 2 minutes, and 72°C for 2 minutes, and final extension at 72°C for 5 minutes. The second round consisted of 94°C for 2 minutes, followed by 5 cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 60°C for 30 seconds, and followed by another 25 cycles of 94°C for 30 seconds, 45°C 30 seconds, and 60°C for 30 seconds, and final extension at 60°C for 2 minutes. For *P. vivax,* the first round consisted of 95°C for 5 minutes, followed by 25 cycles of 94°C for 30

seconds, 52°C for 30 seconds, and 72°C for 30 seconds, and final extension at 72°C for 2 minutes. The second round consisted of 95°C for 5 minutes, followed by 25 cycles of 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 30 seconds, and final extension at 72°C for 2 minutes. Positive and negative controls (*P. falciparum* clone 3D7, *P. vivax, P.malariae, P. ovale* and water) were included in each PCR run.

Interpretation

PCR products were analysed using 3% (w/v) agarose gel electrophoresis, run at 120 volts for 40-70 minutes. A molecular imager Gel Doc XR+ System (BioRad) with the Quantity One 1-D analysis software (BioRad) was used for visualisation, imaging and analysis of the PCR products. DNA band sizes of 159-192bp indicated *P. falciparum* and 128-265bp indicated *P. vivax.* Assays were valid only if the positive and negative controls had appropriate results.

2.9.6 Infection markers

2.9.6.1 Complete blood count (CBC)

Two automated haematology analysers were used at SMRU during the study: A Sysmex pocH-100i (Sysmex Corp., Kobe, Japan) was used between March and April 2011, and a Nihon Kohden celltacF MEK-8222 (Nihon Kohden, Tokyo, Japan) was used between April 2011 and February 2013. The former measured 17 clinical parameters including a 3-part white blood cell (WBC) differential. The latter measured 22 parameters with a 5-part WBC differential. The specimens were processed as part of the routine services at SMRU haematology laboratory and the results were sent back to physicians for patient management as were the CRP results (section 2.9.6.2). The SMRU haematology laboratory also routinely participates in the Thailand NEQAS in Haematology from DMSc: unit code HM 1015.

2.9.6.2 C-reactive protein (CRP)

Acute EDTA blood specimens were tested for the level of CRP, using the NycoCard CRP test (Axis-Shield, Oslo, Norway) following the manufacturer's instructions. Briefly, 5µl of whole blood was added into the R1 dilution liquid tube (containing 400µl of the R1 dilution liquid), and mixed thoroughly for 10 seconds. 50µl of diluted specimen was applied to the test device and allowed to soak into the membrane for 30 seconds. After this, one drop of R2 conjugate was added and allowed to soak for 30 seconds, then one drop of R3 washing solution was added, and allowed to soak for 20 seconds. The result was read within five minutes using the NycoCard Reader II and the CRP whole blood protocol was selected. A correction for haematocrit (Hct) deviating from 40% was required by multiplying the CRP result with the respective factor as shown in the table below (Table 2.10). A reference range of <5 mg/l was indicated in the product description.

Table 2.10 Haematocrit (Hct) correction factor for CRP

Hct (%)	Factor	Hct (%)	Factor
20 – 29	0.8	56 – 58	1.4
30 – 36	0.9	59 – 61	1.5
37 – 42	1.0	62 – 63	1.6
43 – 47	1.1	64 – 65	1.7
48 – 51	1.2	66 – 67	1.8
52 – 55	1.3	68 – 69	1.9

2.10 Data management and analysis

Clinical and laboratory data were recorded on study-specific paper forms and entered in purpose-designed Microsoft Access 2007 databases (Microsoft, Richmond, WA, USA). Clinical data were double-entered into the database and the databases compared to identify data entry errors. Laboratory data were single-entered into the database and all entries were checked against original forms. Statistical analyses were performed using STATA/SE 10.1 (StataCorp LP., College Station, TX, USA) and are described in the appropriate result chapters.

3 Patient demographics, clinical presentations and general laboratory findings

3.1 Introduction

SMRU provides clinical support for migrants and refugees living or working on the Thailand-Myanmar border, of which Karen and Burman are the majority. This chapter describes the characteristics, clinical presentations and laboratory findings of the patients recruited in the study and the comparison between non-malaria and malaria patient groups.

3.2 Materials and Methods

3.2.1 Patients and methods

Febrile patients were recruited into the study from the OPD of two SMRU migrant clinics at Wang Pha and Mawker Thai villages, and one clinic at Mae La refugee camp as previously described in the Materials and Methods chapter, section 2.2-2.6. All patients who presented with fever were screened for malaria by microscopy or RDT. Patients who were negative for malaria screen using either method were recruited into PART I of the study (non-malaria patients) and patients who had a positive result were recruited into PART II (malaria patients). From this chapter onward, the term migrants refers to patients from Wang Pha and Mawker Thai clinics while refugees refers to patients from the clinic at Mae La camp. Adults refers to patients aged at least 18 years and children refers to patients aged between five and 17 years old.

3.2.2 Statistical analysis

Data collection was performed as described in the Materials and Methods chapter, section 2.6. The data were analysed using STATA/SE 10.1 (StataCorp LP) and graphs were drawn using Microsoft Excel 2010 (Microsoft). Numeric data were described by their medians and interquartile ranges (IQR) or ranges as appropriate. Non-parametric

continuous variables were compared using Wilcoxon rank-sum (Mann Whitney U) test. Categorical variables were compared using the Chi-squared (χ^2) test, unless otherwise stated.

3.3 Results

The study was conducted between March 2011 and March 2013, with a total of 1,029 patients recruited from all three OPD clinics: 908 patients were recruited into PART I (non-malaria patients) and 121 patients were recruited into PART II (malaria patients). The study did not define which temperature measurement method to use. The method used was based on routine practice at each clinic and depended on the discretion of the clinicians/medics. The chosen method was recorded in the study CRF. Most patients, 95.8% (986/1,029), had temperature measured at presentation by the tympanic method, 2.5% (26/1,029) of patients were measured by the axillary method, 1.5% (15/1,029) were measured by the temporal method and 0.2% (2/1,029) by the rectal method.

Of these, 46.7% (481/1,029) were from the two migrant clinics; 349 patients from Wang Pha and 132 patients from Mawker Thai, and 53.3% (548/1,029) were from the clinic at Mae La refugee camp. The overall median age of the patients was 19 years (IQR 12-30, range 5-63), 42.5% (437/1,029) of the patients were children and 63.9% (657/1,029) of the patients were male. The overall follow-up rate was high, with 89.2% (918/1,029) of the patients returning for a follow-up visit.

3.3.1 PART I (non-malaria patients)

3.3.1.1 Patient demographics

Of the 908 non-malaria patients recruited, 55.7% (506/908) of the patients were from the refugee clinic and 61.8% (561/908) were male (Table 3.1). The number of patients recruited from migrant and refugee clinics over the two years of the study period by month is shown in Figure 3.1. Patients were recruited throughout the study period

peaking for both migrant and refugee populations during the rainy season (May-October). The proportion of males was the same for both migrant and refugee populations (Table 3.1). The median age of the patients was 18 years (IQR 12-30, range 5-63) and 45.2% (410/908) of patients were children. The number of patients recruited from migrants and refugees by age groups is shown in Figure 3.2. The number of male patients was higher than female patients in almost all age groups apart from patients aged between 36-40 years (Figure 3.3). The migrant patient group was slightly older than the refugee patient group with the median age of 20 years (IQR 12-33) compared to 18 years (IQR 12-27) (*P*=0.022). There were no differences in the proportion of migrant and refugee child patients recruited (*P*=0.051). The follow-up rate was high with 89.4% (812/908) and more patients from the refugee clinic were seen at the follow-up visit compared to migrant clinics (95.1 vs. 82.3%, *P*=<0.001). The median interval duration between enrolment and follow-up visit was 14 days (IQR 14-14), with a range of 9-29 days.

Table 3.1 Demographic data of 908 non-malaria patients, comparing migrant and refugee populations.

General characteristics	Number of patients (%)			*P*-value
	All patients	**Migrants**	**Refugees**	
Recruited	908 (100)	402 (44.3)	506 (55.7)	
Sex: male	561 (61.8)	250 (62.2)	311 (61.5)	0.823
Median (IQR) age, years	**18 (12-30)**	**20 (12-33)**	**18 (12-27)**	**0.022**
Children	410 (45.2)	167 (41.5)	243 (48.0)	0.051
Follow-up rate	**812 (89.4)**	**331 (82.3)**	**481 (95.1)**	**<0.001**

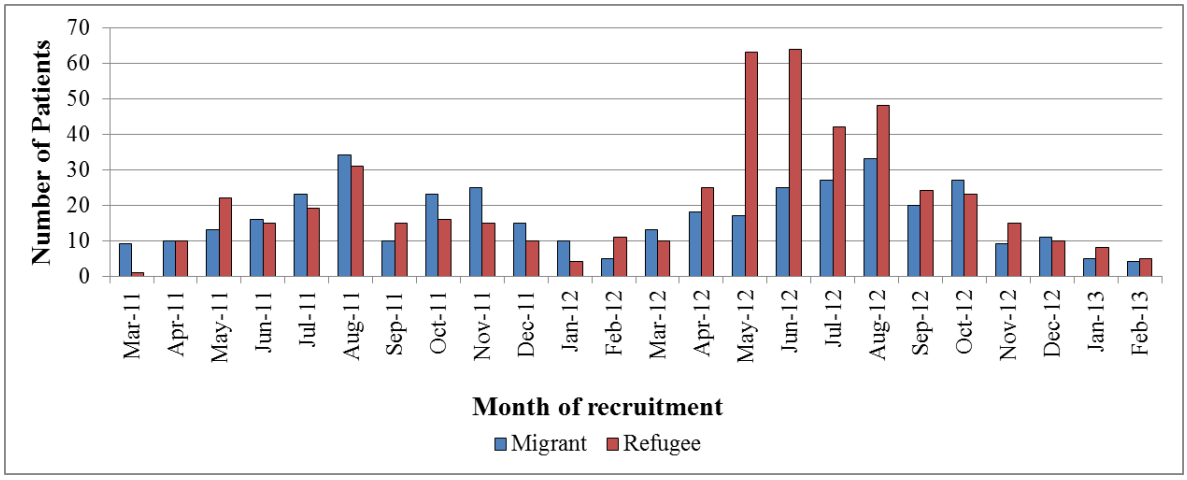


Figure 3.1 Number of non-malaria patients recruited from migrants and refugees by month.

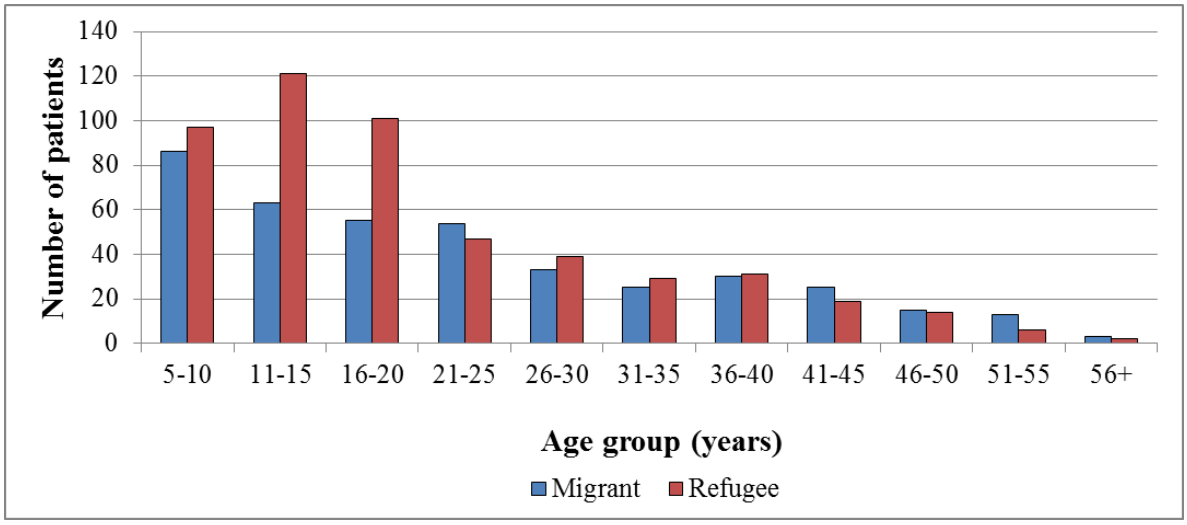


Figure 3.2 Number of non-malaria patients recruited from migrants and refugees by age group.

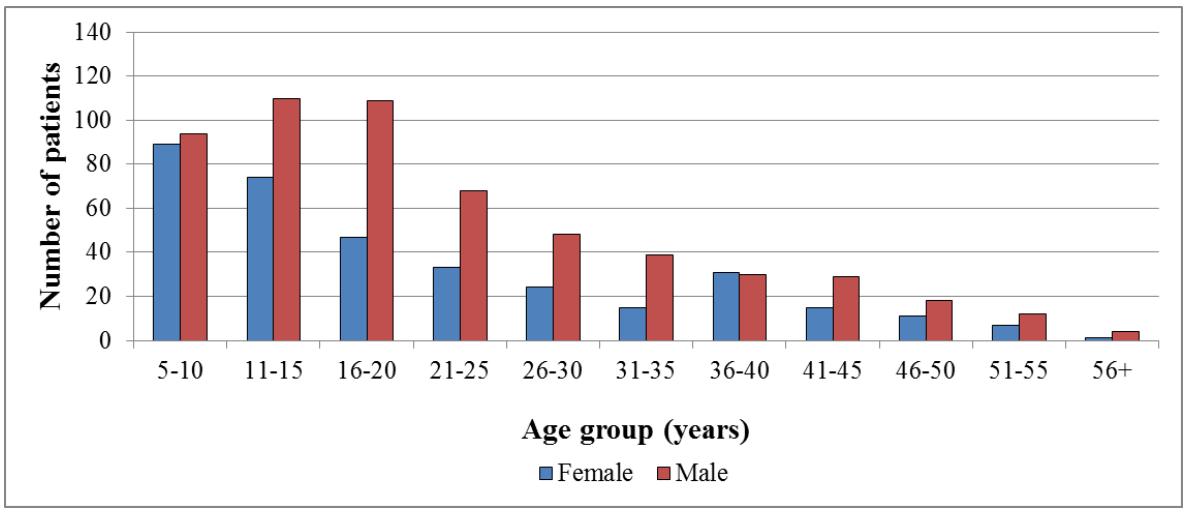


Figure 3.3 Number of non-malaria patients recruited by age and sex.

3.3.1.2 Clinical presentations and laboratory findings

The clinical presentations and laboratory findings of 908 non-malaria patients recruited in the study are summarised in Table 3.2. The median duration of fever at presentation was two days (IQR 2-3), with a range of 1-7 days. The median temperature was 38.5°C (IQR 38.2-39.0), with a range of 38.0-40.9°C. Patients presented early in the course of their illness with most patients reporting having symptoms for two days. Headache was the most frequent symptom reported (91.1%), followed by joint pain (55.8%) and muscle pain (43.2%). Skin rashes, jaundice and abnormal bleeding were uncommon. General laboratory findings were also recorded and analysed including CBC and CRP as shown in Table 3.2.

Table 3.2 Clinical presentations and laboratory findings of 908 non-malaria febrile patients.

Clinical presentations	Number of patients (%)	Median (range)
Heart rate (beats/minute)	-	96 (60-140)
Respiratory rate (breaths/minute)	-	26 (18-48)
Temperature (°C)	-	38.5 (38.0-40.9)
Capillary refill time (second)	-	2 (1-2)[a]
Blood pressure (mmHg)		
• Systolic	815 (89.8)	100 (70-150)
• Diastolic	815 (89.8)	70 (40-100)
Fever	908 (100)	-
Headache	827 (91.1)	-
Joint pain	507 (55.8)	-
Muscle pain	392 (43.2)	-
Cough	366 (40.3)	-
Red eyes	276 (30.4)	-
Lymph nodes palpable	237 (26.1)	-
Abdominal pain	197 (21.7)	-
Constipation	160 (17.6)	-
Pain behind eyes	129 (14.2)	-
Stiff neck	60 (6.6)	-

Table 3.2 Clinical presentations and laboratory findings of 908 non-malaria febrile patients (continued).

Clinical presentations	Number of patients (%)	Median (range)
Skin rash	32 (3.5)	-
• Petechiae	19 (59.4)	-
• Eschar	2 (6.3)	-
• Maculopapular rash	1 (3.1)	-
• Other types of rash	10 (31.3)	-
Jaundice	22 (2.4)	-
Abnormal bleeding	9 (1.0)	-
Laboratory findings		
White blood cells (10^3/μl)	-	7.1 (1.5-30.3)[b]
• Neutrophils (10^3/μl)	-	5.2 (0.8-25.8)[c]
• Lymphocytes (10^3/μl)	-	0.9 (0-6.6)[d]
Haematocrit (%)	-	40.2 (18.3-77.2[e])[b]
Platelets (10^3/μl)	-	214 (30-615)[b]
C-reactive protein (mg/l)	-	20.7 (4.5-230.4)[b]

[a]Data available in 903/908 (99.4%) cases, [b]Data available in 907/908 (99.9%) cases, [c]Data available in 904/908 (99.6%) cases, [d]Data available in 905/908 (99.7%) cases, [e]A haematocrit of 77.2% was obtained for a single patient. This is abnormally high and therefore likely to be an error.

3.3.2 PART II (malaria patients)

3.3.2.1 Patient demographics

Of the 121 malaria patients recruited, 34.7% (42/121) of the patients were from the refugee clinic and 79.3% (96/121) were male (Table 3.3). The number of malaria patients recruited from migrants and refugees over the recruitment period by month is shown in Figure 3.4. There were more patients recruited from migrants than refugees throughout most of the recruitment period. The proportions of male and female were not different

between migrants and refugees (Table 3.3). The median age of patients was 23 years (IQR 18-34, range 5-58), while 22.3% (27/121) of patients were children. The number of patients recruited from migrants and refugees by age groups is shown in Figure 3.5. There were more patients recruited from migrants than refugees in almost all age groups apart from patients aged between 5-15 years. The number of male patients was higher than female in almost all age groups apart from patients aged between 5-10 years (Figure 3.6). Patients from migrant clinics were older than from the refugee clinic with the median age of 25 years (IQR 19-36) compared to 20 years (IQR 15-20) (P=0.006). There were more child patients from refugees than from migrants (33.3 vs. 16.5%, P=0.034). The follow-up rate was high with 87.6% (106/121) of the patients returning for the follow-up visit. The rate was not significantly different between migrants and refugees (83.5 vs. 95.2%, P=0.083, Fisher's exact test). The median interval duration between enrolment and follow-up visit was 14 days (IQR 14-14), with a range of 11-48 days.

Table 3.3 Demographic data of 121 malaria patients comparing migrant and refugee populations.

General characteristics	**Number of patients (%)**			***P*-value**
	All patients	**Migrants**	**Refugees**	
Recruited	121 (100)	79 (65.3)	42 (34.7)	
Sex: male	96 (79.3)	66 (83.5)	30 (71.4)	0.117
Median (IQR) age, years	**23 (18-34)**	**25 (19-36)**	**20 (15-25)**	**0.006**
Children	**27 (22.3)**	**13 (16.5)**	**14 (33.3)**	**0.034**
Follow-up rate	106 (87.6)	66 (83.5)	40 (95.2)	0.083*

*Fisher's exact test

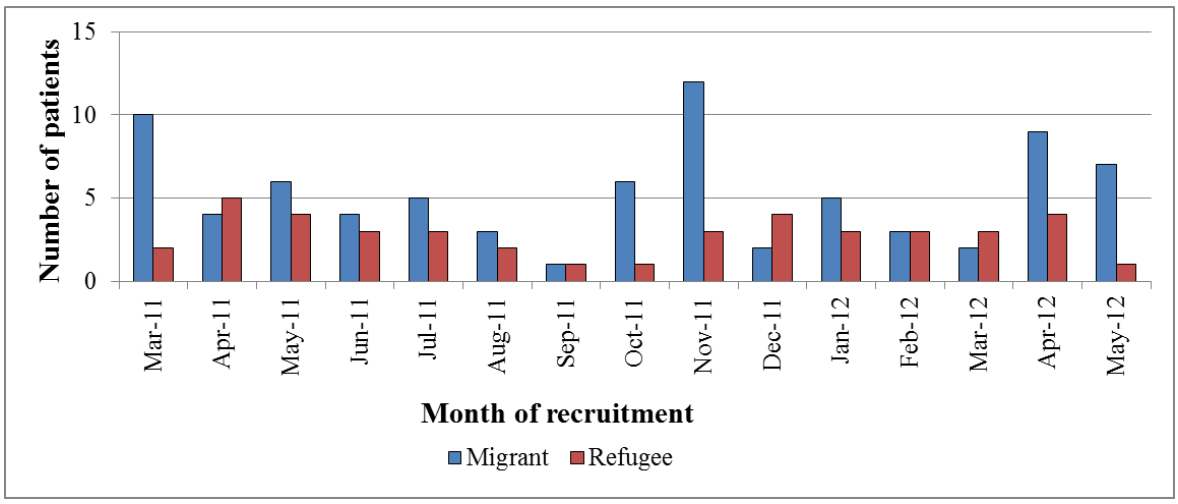


Figure 3.4 Number of malaria patients recruited from migrants and refugees by month.

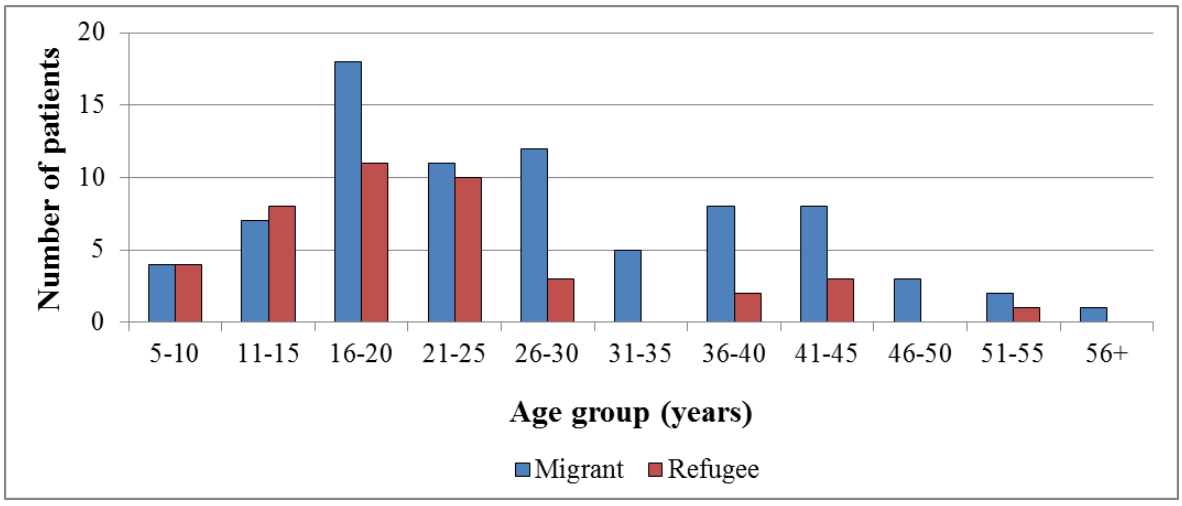


Figure 3.5 Number of malaria patients recruited from migrants and refugees by age group.

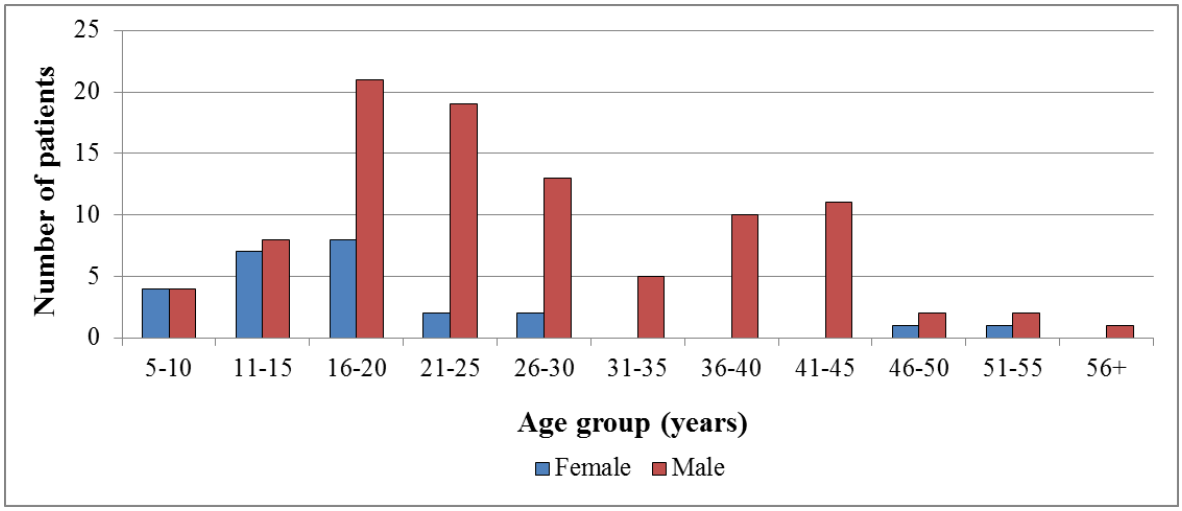


Figure 3.6 Number of malaria patients recruited by age and sex.

3.3.2.2 Clinical presentations and laboratory findings

The clinical presentations and laboratory findings of 121 malaria patients recruited in the study are summarised in Table 3.4. The median duration of fever at presentation was three days (IQR 2-3), with a range of 1-7 days. The median temperature was 39.0°C (IQR 38.4-39.6), with a range of 38.0-40.6°C. Malaria patients also presented to the clinics early in the course of their fever with most patients reporting having symptoms for two or three days. Headache was the most common symptom (96.7%), followed by joint pain (66.1%), and muscle pain (43.0%). Red eyes and abnormal bleeding were uncommon. None of the patients reported skin rashes.

Table 3.4 Clinical presentations and laboratory findings of 121 malaria patients.

Clinical presentations	Number of patients (%)	Median (range)
Heart rate (beats/minute)	-	95 (60-140)
Respiratory rate (breaths/minute)	-	24 (20-44)
Temperature (°C)	-	39.0 (38.0-40.6)
Capillary refill time (second)	-	2 (1-2)
Blood pressure (mmHg)		
• Systolic	116 (95.9)	110 (80-140)
• Diastolic	116 (95.9)	70 (40-90)
Fever	121 (100)	-
Headache	117 (96.7)	-
Joint pain	80 (66.1)	-
Muscle pain	52 (43.0)	-
Cough	38 (31.4)	-
Constipation	24 (19.8)	-
Abdominal pain	22 (18.2)	-
Pain behind eyes	19 (15.7)	-
Stiff neck	11 (9.1)	-
Jaundice	10 (8.3)	-
Lymph nodes palpable	8 (6.6)	-

Table 3.4 Clinical presentations and laboratory findings of 121 malaria patients (continued).

Clinical presentations	Number of patients (%)	Median (range)
Red eyes	7 (5.8)	-
Abnormal bleeding	1 (0.8)	-
Skin rash	0 (0)	-
Laboratory findings		
White blood cells (10^3/μl)	-	5.3 (1.4-17.3)
• Neutrophils (10^3/μl)	-	3.9 (1.0-14.2)[a]
• Lymphocytes (10^3/μl)	-	0.6 (0.1-3.0)[b]
Haematocrit (%)	-	39.1 (21.9-57.4)
Platelets (10^3/μl)	-	97 (19-485)
C-reactive protein (mg/l)	-	63.0 (7.2-200.1)

[a]Data available in 118/121 (97.5%) cases, [b]Data available in 120/121 (99.2%) cases

3.3.3 Comparison of patient demographics, clinical presentations and laboratory findings for PART I (non-malaria patients) and PART II (malaria patients) of the study

A comparison of demographics, clinical presentations and laboratory findings between the malaria and non-malaria patients are shown in Table 3.5**Error! Reference source not found.**. The median age of patients presenting with malaria was higher than those presenting without malaria (23 vs. 18 years, P<0.001) and the proportion of child patients was also smaller in the malaria patients (45.2 vs 22.3%, P<0.001). The proportion of male patients recruited was higher in the malaria group than the non-malaria group (79.3 vs. 61.8%, P<0.001), and the proportion of refugee patients was higher in the non-malaria group (55.6 vs. 34.7%, P<0.001). The follow-up rate was similar for both malaria and non-malaria groups.

Malaria patients reported longer duration of fever (three vs. two days, P=0.001) and higher presenting temperature (39.0 vs. 38.5°C, P<0.001) than non-malaria patients. The non-malaria patients reported significantly higher occurrence of red eyes, skin rash, and palpable lymph nodes whereas malaria patients reported higher occurrence of headache, jaundice, and joint pain (Table 3.5).

For the general laboratory findings, the median CRP concentration was much higher in patients presenting with malaria compared to non-malaria fever (63.0 vs. 20.7 mg/l, P<0.001) whereas the platelet count was much lower (97 vs. 214 x10^3/µl, P<0.001). The WBC count and haematocrit were slightly lower in malaria patients compared to the non-malaria patients (Table 3.5).

Table 3.5 Comparison of patient demographics, clinical presentations and laboratory findings for non-malaria and malaria patients (n=1,029).

	Non-malaria patients (n=908)	Malaria patients (n=121)	*P*-value
Age (years)	**18.0 (IQR 12.0-30.0)**	**23.0 (IQR 18.0-34.0)**	**<0.001**
Children	**410/908 (45.2%)**	**27/121 (22.3%)**	**<0.001**
Sex: male	**561/908 (61.8%)**	**96/121 (79.3%)**	**<0.001**
Location: refugee clinic	**506/908 (55.7%)**	**42/121 (34.7%)**	**<0.001**
Follow-up rate	812/908 (89.4%)	106/121 (87.6%)	0.543
Days of fever	**2 (IQR 2-3)**	**3 (IQR 2-3)**	**0.001**
Presenting temperature (°C)	**38.5 (IQR 38.2-39.0)**	**39.0 (IQR 38.4-39.6)**	**<0.001**
Headache	**827/908 (91.1%)**	**117/121 (96.7%)**	**0.034**[f]
Stiff neck	60/908 (6.6%)	11/121 (9.1%)	0.311
Pain behind the eyes	129/908 (14.2%)	19/121 (15.7%)	0.660
Red eyes	**276/908 (30.4%)**	**7/121 (5.8%)**	**<0.001**
Jaundice	**22/908 (2.4%)**	**10/121 (8.3%)**	**0.001**
Muscle pain	392/908 (43.2%)	52/121 (43.0%)	0.967
Joint pain	**507/908 (55.8%)**	**80/121 (66.1%)**	**0.032**
Cough	366/908 (40.3%)	38/121 (31.4%)	0.060

Table 3.5 Comparison of patient demographics, clinical presentations and laboratory findings for non-malaria and malaria patients (n=1,029) (continued).

	Non-malaria patients (n=908)	Malaria patients (n=121)	*P*-value
Constipation	160/908 (17.6%)	24/121 (19.8%)	0.551
Abdominal pain	197/908 (21.7%)	22/121 (18.2%)	0.375
Abnormal bleeding	9/908 (1.0%)	1/121 (0.8%)	1.000[f]
Skin rash	**32/908 (3.5%)**	**0/121 (0%)**	**0.026[f]**
Lymph nodes palpable	**237/908 (26.1%)**	**8/121 (6.6%)**	**<0.001**
White blood cell (10^3/µl)	**7.1 (IQR 5.2-10.0)[a]**	**5.3 (IQR 4.1-6.8)**	**<0.001**
• **Neutrophils (10^3/µl)**	**5.2 (IQR 3.5-7.7)[b]**	**3.9 (IQR 3.0-5.0)[d]**	**<0.001**
• **Lymphocytes (10^3/µl)**	**0.9 (IQR 0.6-1.4)[c]**	**0.6 (IQR 0.5-0.9)[e]**	**<0.001**
Haematocrit (%)	**40.2 (IQR 37.2-43.4)[a]**	**39.1 (IQR 35.9-42.6)**	**0.015**
Platelets (10^3/µl)	**214.0 (IQR 167.0-268.0)[a]**	**97.0 (IQR 68.0-129.0)**	**<0.001**
C-reactive protein (mg/l)	**20.7 (IQR 8.0-58.5)[a]**	**63.0 (IQR 34.0-96.0)**	**<0.001**

[a]Data available in 907/908 (99.9%) cases, [b]Data available in 904/908 (99.6%) cases, [c]Data available in 905/908 (99.7%) cases, [d]Data available in 118/121 (97.5%) cases, [e]Data available in 120/121 (99.2%) cases, [f]Fisher's exact test

3.4 Discussion

This study was a two-year exploration of the causes of non-malaria fever and a year exploration of co-infection with malaria fever in patients presenting to SMRU clinics on the Thailand-Myanmar border. The overall proportion of patient recruitment was similar between migrant and refugee populations with a high follow-up rate. Patients presented to SMRU clinics early in the course of their illness with the median duration of fever of two days (IQR 2-3) for non-malaria patients and three days (IQR 2-3) for malaria patients. The difference in the time of presentation to the clinics was not statistically significant between refugee and migrant populations for both patient groups. CRP concentration and platelet count were clearly different between the malaria and non-malaria patients. The median CRP concentration was much higher in malaria patients compared to non-malaria patients. This could be due to viral infections in the non-malaria patient group [268]. Thrombocytopenia is known to be a common haematological presentation associated with acute malaria infection [269, 270]. This study also revealed that the platelet count was much lower in the malaria patients compared to non-malaria patients. Several clinical presentations and laboratory findings were significantly different between malaria and non-malaria patients. Further analyses of those relating to the diagnosis of infections will be described in the following chapter.

There were some differences in the patient demographics between malaria and non-malaria patient groups. The number of patients recruited from the refugee clinic was slightly higher than those from migrant clinics for the non-malaria patients (55.7 vs. 44.3%) whereas the number of patients recruited from migrant clinics was almost two times higher than those from the refugee clinic in the malaria patients (65.3 vs. 34.1). This reflects the recruitment strategy for malaria patients, which was to recruit, every week, the first eligible patient from each site (Mae La, Wang Pha and Mawker Thai) i.e. one refugee

and two migrant patients. The slightly higher numbers of refugees recruited with non-malaria fever compared with migrants is likely to be multi-factorial influenced by distance from clinic and higher incidence rates of malaria in locations inhabited by migrants [252]. It should also be noted that SMRU is well known for malaria research and treatment, and therefore when patients have fever, but they do not think that they have malaria, they are more likely to go to other non-SMRU clinics.

There were more male than female patients in both non-malaria (61.8% male) and malaria (79.3% male) groups. The proportion of males was higher for malaria patients compared to non-malaria patients ($P<0.001$), however, the proportion was not significantly different between migrants and refugees for both groups. Migrants were significantly older than refugees for both malaria and non-malaria patients ($P=0.006$ and 0.022, respectively). The proportion of children was significantly larger in refugees ($P=0.034$) for malaria patients, however, it was not statistically different for non-malaria patients. This may be because the older male migrants work more in the field and when they are sick they come to the clinics on the way to work. Therefore, they are more likely to seek health care than young children, whereas the young child refugees are living in the camp closer to the clinic. The follow-up rates were similarly high for both groups, which could be explained by the excellent community participation in SMRU research and a reminder system to follow-up patients was also in place i.e. a study follow-up card was given at enrolment or calls were made prior to appointment. Further analyses of demographic data relating to the diagnosis of infections will also be described in the following chapter.

4 Causes of acute undifferentiated febrile illness on the Thailand-Myanmar border

4.1 Introduction and aims

Acute undifferentiated febrile illness (AUFI) is common in SE Asia. Identifying the causative agents of AUFI is difficult using either clinical presentations or laboratory testing because the common causes of AUFI have non-specific symptoms, and resources do not exist to allow comprehensive laboratory interrogation of clinical specimens. With a substantial decrease of malaria in SE Asia, the need for understanding the other causes of AUFI has increased. The aims of this study were to:

1. Estimate the occurrence of common non-malaria causes of AUFI, including dengue, leptospirosis, rickettsial infections (focusing on scrub typhus and murine typhus), and other invasive bacterial infections in the SMRU clinic populations (migrants and refugees) and improve understanding of these infections in the population to assist the development of the treatment algorithms and patient management of non-malaria fever cases.

2. Assess the impact of dual infection with malaria:

    a. Quantify and detect sub-microscopic malaria infections in febrile patients

4.2 Materials and Methods

4.2.1 Patients and methods

All recruited patients from the study: 908 non-malaria febrile patients (PART I) and 121 malaria patients (PART II) were included in the analysis. Diagnosis was based on the results of gold standard diagnostic tests. These included serological assays for diagnosis of dengue/JE, murine typhus, and scrub typhus, and blood culture for other bacterial infections.

Patients with available paired acute and convalescent serum specimens were tested using serological assays. Detection of IgM/IgG antibodies against dengue virus and JE virus by ELISAs (AFRIMS) was used to determine dengue and JE virus infection status [70, 258]. ELISA and IFA (MORU) were used to detect IgM antibodies against *O. tsutsugamushi* and *R. typhi* for diagnosis of scrub typhus and murine typhus [207, 263]. Blood cultures from all patients at enrolment were used to determine other causes of invasive bacterial infections. The qPCR targeting the 16S *rRNA* gene was used to investigate leptospirosis from all acute plasma specimens since the serological gold standard test, MAT, was not available at the time of the study [147, 149, 150]. The diagnostic tests described above were considered to be reference diagnostic tests for the final diagnosis. All diagnoses in this study refer to acute infections.

In addition to the malaria microscopy (malaria smear) and RDT (SD BIOLINE Malaria Antigen P.f/ Pan POCT, Standard Diagnositcs, Inc.) that were used to screen febrile patients in the clinics at the enrolment visit, the highly sensitive qPCR targeting 18S *rRNA* gene specific for *Plasmodium* species [241, 249] was performed to detect sub-microscopic malaria infection from all non-malaria febrile patients using acute packed red cell specimens. For specimens that were positive by 18S *rRNA* qPCR assay, the malaria parasite species were identified using microsatellite nested-PCR assays specific to *P.*

falciparum (microsatellite marker PFPK2) and *P. vivax* (microsatellite marker 3.502) [266, 267]. Specimens were reported as indeterminate species when there was no amplification obtained in the nested-PCR assays or insufficient DNA to perform the assays.

A CBC and CRP measurement were performed on all acute blood specimens. The details of all laboratory methods and their interpretations are described in the Materials and Methods chapter, section 2.9.

4.2.2 Data analysis

The data were analysed using STATA/SE 10.1 (StataCorp LP) and the graphs were created using STATA/SE 10.1 (StataCorp LP), Microsoft Excel 2010 (Microsoft), and GraphPad Prism 7.02 (GraphPad Software Inc., CA, USA). Numeric data were described by medians and interquartile ranges (IQR) or means and confidence intervals (CI), as appropriate. Demographics, clinical presentations and general laboratory findings were described for cases and non-cases of each diagnosis (dengue, leptospirosis, murine typhus and scrub typhus). Variables including demographics, clinical presentations, and general laboratory findings were assessed (Table 4.1), using univariate logistic regression, to determine whether they were associated with the final diagnosis of dengue, leptospirosis, murine typhus or scrub typhus.

Table 4.1 Variables tested for univariate logistic regression.

Variables	
Age (year)	Days of joint pain
Sex: male	Cough
Location: refugee clinic	Days of cough
Presenting temperature (°C)	Constipation
Days of fever	Days of constipation
Headache	Abdominal pain
Days of headache	Days of abdominal pain
Stiff neck	Abnormal bleeding
Days of Stiff neck	Days of abnormal bleeding
Pain behind the eyes	Skin rash
Days of pain behind the eyes	Days of skin rash
Red eyes	Lymph node palpable
Days of red eyes	White blood cell (10^3/µl)
Jaundice	Neutrophils (10^3/µl)
Days of jaundice	Lymphocytes (10^3/µl)
Muscle pain	Haematocrit (%)
Days of muscle pain	Platelets (10^3/µl)
Joint pain	C-reactive protein (mg/l)

Variables that were significant in the univariate logistic regression were selected to be considered in the multivariable (multivariate) logistic regression analysis to discover demographics, clinical presentations and laboratory findings that were independently associated with each disease (i.e. to adjust or control for confounding of the variables). A 95% CI for the odds ratio (OR) was reported. All tests were performed at 5% significance

level. A stepwise regression technique was used to select variables that were independently associated with each diagnosis. The probability of entry was set at 0.05 and the probability of removal was set at 0.055 [271]. Stepwise multivariate logistic regression was performed on all variables that were significant from univariate logistic regression, except WBC differentials. If the total WBC count was significant in the multivariate model, then it was considered further by expanding the WBC count into differentials to see which were having more impact. Non-parametric continuous variables were compared using the Wilcoxon rank-sum (Mann Whitney U) test or Kruskal-Wallis equality of population rank test, as appropriate. Proportions of malaria qPCR positive specimens were compared between groups using two-sample test of proportions (prtest). Geometric means were compared using t-test or ANOVA as appropriate. The geometric means of sub-microscopic malaria parasitaemia by qPCR were compared between groups using logistic regression of log data, followed by ANOVA or pairwise comparison, as appropriate. The tests were considered statistically significant with *P*-value less than 0.05.

4.3 Results: non-malaria febrile patients (PART I)

4.3.1 Diagnosis of non-malaria causes of fever

A total of 908 non-malaria patients were enrolled into the study between March 2011 and February 2013. Diagnoses of these febrile patients were based on the results of the reference diagnostic tests and are shown in Figure 4.1. The figure demonstrates the number of patients and percentage for each diagnosis. Dengue was the most common cause of fever in this cohort accounting for 15.9% (144/908) of the patients, followed by 6.0% (54/908) with leptospirosis, 6.0% (54/908) with murine typhus, 3.2% (29/908) with scrub typhus, 1.5% (14/908) co-infection of leptospirosis and scrub typhus, 1.2% (11/908) with significant bacterial infections (Table 4.2), 0.7% (6/908) with JE, and 0.1% (1/908) co-infection with *Streptococcus pneumoniae* bacteraemia and scrub typhus. The causes of

fever remained unknown in 65.5% (595/908) of the patients. The overall co-infection rate in this study was found to be 1.7% (15/908) of the patients.

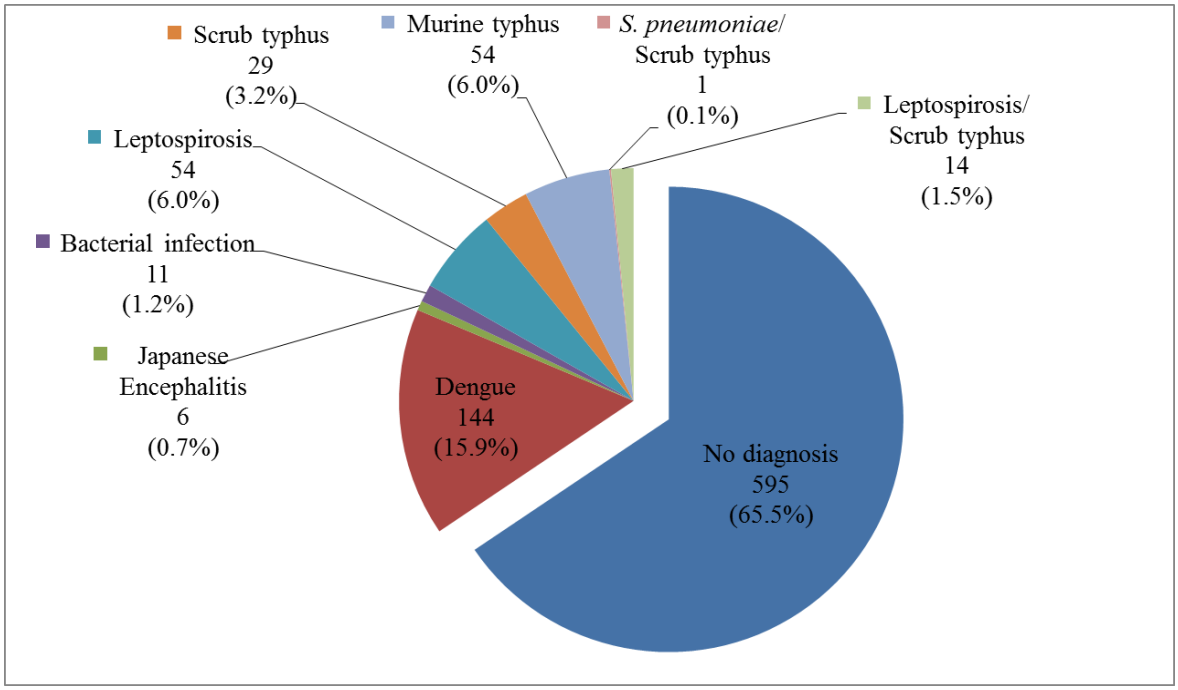


Figure 4.1 Diagnosis of 908 non-malaria febrile patients based on the results of the reference diagnostic tests.

Patients with invasive bacterial infections were determined according to the blood culture results. Blood culture was performed according to the SMRU blood culture SOP (MBL-2-B). *Staphylococcus* sp. (coagulase negative), diphtheroids, or *Bacillus* sp. were regarded as likely contaminants. The significance of all other organisms grown were discussed between the clinical microbiologist and the clinician. Of the 908 patients tested, only 1.3% (12/908) were positive for significant pathogens, in which *Escherichia coli* was the most common (0.6%, 5/908), followed by *Salmonella* Typhi (0.2%, 2/908) and one patient of each organism (0.1%) including *Enterococcus* sp., *Moraxella catarrhalis, Salmonella* Paratyphi A, *Salmonella* sp., and *Streptococcus pneumoniae* (Table 4.2). Of these patients with bacterial infections, one patient had concomitant *Streptococcus pneumoniae* bacteraemia and scrub typhus.

Table 4.2 Blood culture results detailing significant organisms.

Organisms	Number of patient (%)
No growth at five days	828 (91.2)
Contaminants[a]	68 (7.5)
Escherichia coli	5 (0.6)
Salmonella Typhi	2 (0.2)
Enterococcus sp.	1 (0.1)
Moraxella catarrhalis	1 (0.1)
Salmonella Paratyphi A	1 (0.1)
Salmonella sp.	1 (0.1)
Streptococcus pneumoniae[b]	1 (0.1)
Total	908 (100.0)

[a]53 patients with growth of *Staphylococcus* sp. (coagulase negative), nine patients with *Bacillus* sp., four patients with diphtheroids, one patient with *Pseudomonas oryzihabitans*, and one patient with *Streptococcus* sp. (alpha haemolytic); [b]co-infection with scrub typhus

4.3.2 Occurrence of non-malaria causes of fever in the clinic populations (migrants and refugees).

The proportions of patients from migrant and refugee clinics by laboratory confirmed diagnosis are shown in Figure 4.2. The numbers of diagnoses are low when stratified by migrant and refugee clinic populations and were too small to perform useful statistical analysis. However, the overall result showed that the proportion of confirmed diagnoses was higher in the patients from the refugee clinic than in migrant clinics. The proportion of patients with dengue, leptospirosis and murine typhus were much higher in refugees than migrants (23.5 vs. 6.2, 7.9 vs. 3.5, and 7.5 vs. 4.0%, respectively). Both

refugees and migrants reported similar proportions of scrub typhus (3.4 vs. 3.0%), invasive bacterial infections (1.2 vs. 1.2%), JE (0.8 vs. 0.5%), and concomitant leptospirosis and scrub typhus (1.4 vs. 1.7%). Only one case of concomitant *S. pneumoniae* bacteraemia and scrub typhus was reported from the migrants. Hence, the proportion of patients with unknown diagnosis was higher in the migrants compared to refugees (79.6 vs. 54.3%).

Only one patient died (0.1%). A 35 year-old man presented to the clinic in Mae La refugee camp with fever of 38.0°C after one day onset of fever, headache, red-eyes, muscle pain and joint pain. He was diagnosed with unknown fever at the time of presentation. The empiric treatment was paracetamol (500mg/tablet) 1¾ tablets, four times daily for five days. Four days later, he presented to the SMRU clinic again with septic shock and fever. He was hospitalised and was given an intravenous normal saline bolus, ceftriaxone intravenous one gram, and oxygen 10 litres/minute by facemask. He was referred urgently to the Première Urgence-Aide Médicale Internationale (PU-AMI) clinic in Mae La refugee camp. Despite this treatment, he died the next morning. Blood culture for samples collected at presentation and the second visit were both negative. No diagnosis of the causative agent of fever was made at the time, but subsequently, the acute specimens were tested by all reference laboratory tests (see section 4.2.1) and found positive for leptospirosis by qPCR tested on acute frozen plasma specimen.

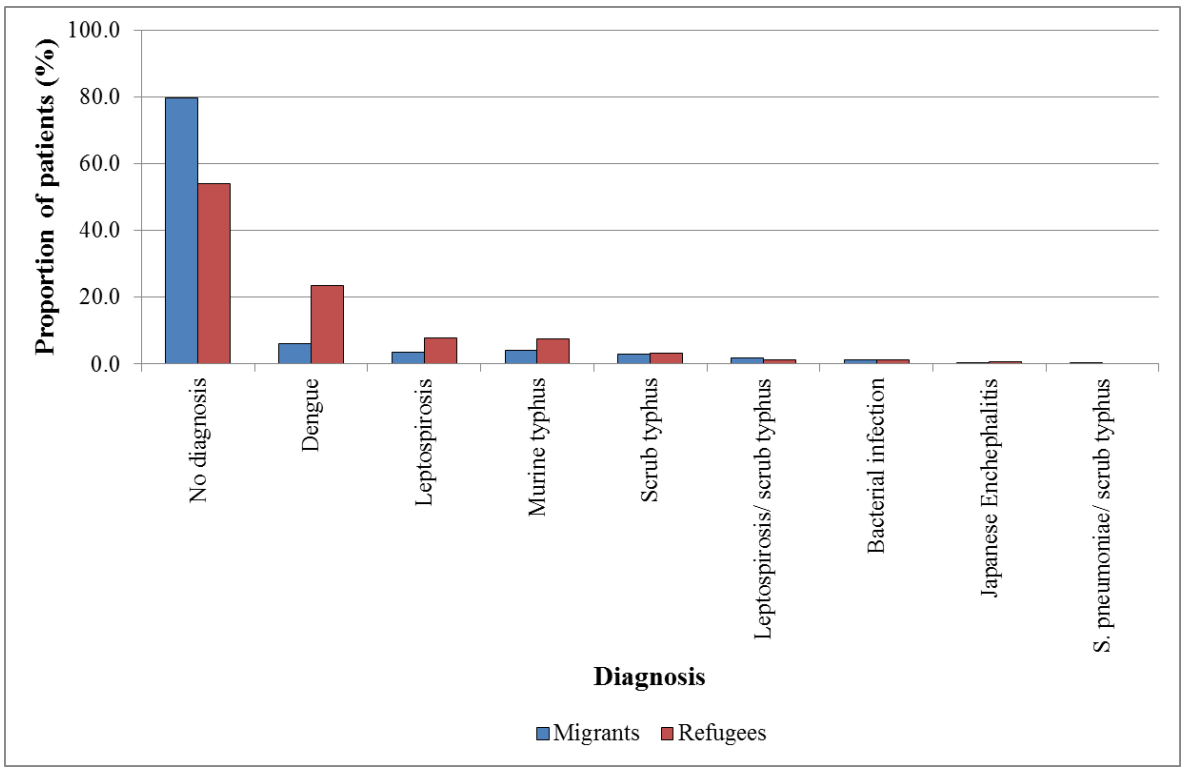


Figure 4.2 Proportion of patients from migrant and refugee clinics by laboratory confirmed diagnosis.

4.3.2.1 Age distribution of dengue, leptospirosis, murine typhus and scrub typhus patients

Age distributions of patients with laboratory confirmed diagnosis of dengue, leptospirosis, murine typhus and scrub typhus are shown in Figure 4.3. Patients with dengue and leptospirosis diagnosis were younger and mostly reported in patients aged between five and 20 years, whereas patients with murine typhus and scrub typhus were reported consistently in almost all age groups.

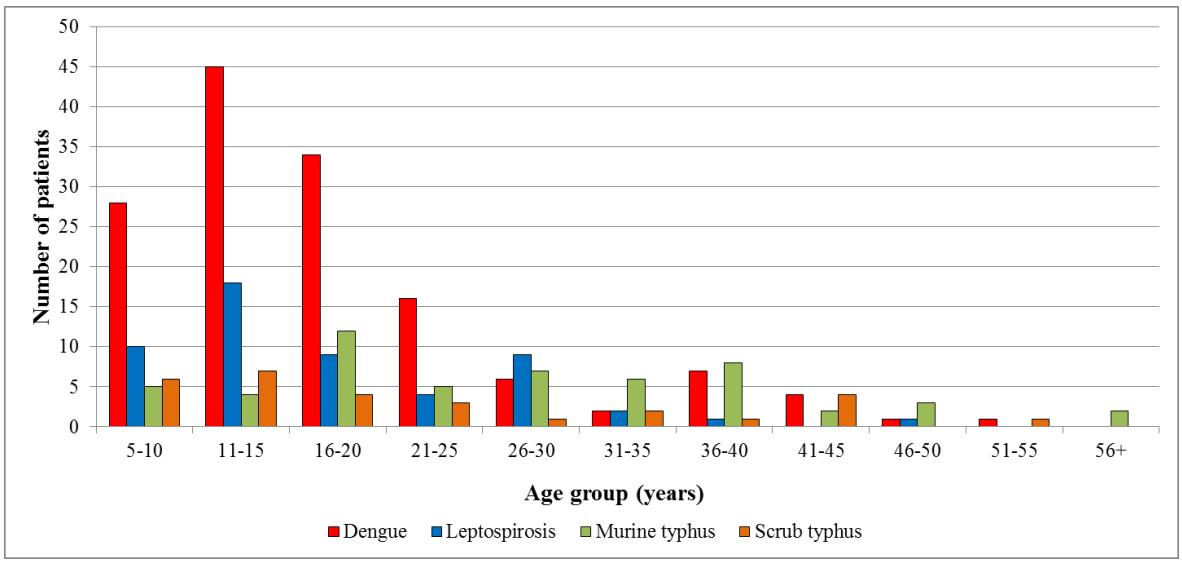


Figure 4.3 Age distribution of patients with laboratory confirmed diagnosis of dengue, leptospirosis, murine typhus and scrub typhus.

4.3.3 Seasonality of dengue, leptospirosis, murine typhus and scrub typhus

The seasonality of dengue, leptospirosis, murine typhus, and scrub typhus by month over the study period of two years (March 2011-March 2013) is shown in Figure 4.4. A small number of dengue cases were reported during 2011, then there was an outbreak of dengue in 2012, peaking between May and July. Leptospirosis cases were reported mostly during the rainy season between July and October, whereas murine typhus and scrub typhus cases were found throughout the year. There was no clear evidence of seasonality for murine typhus and scrub typhus in this study.

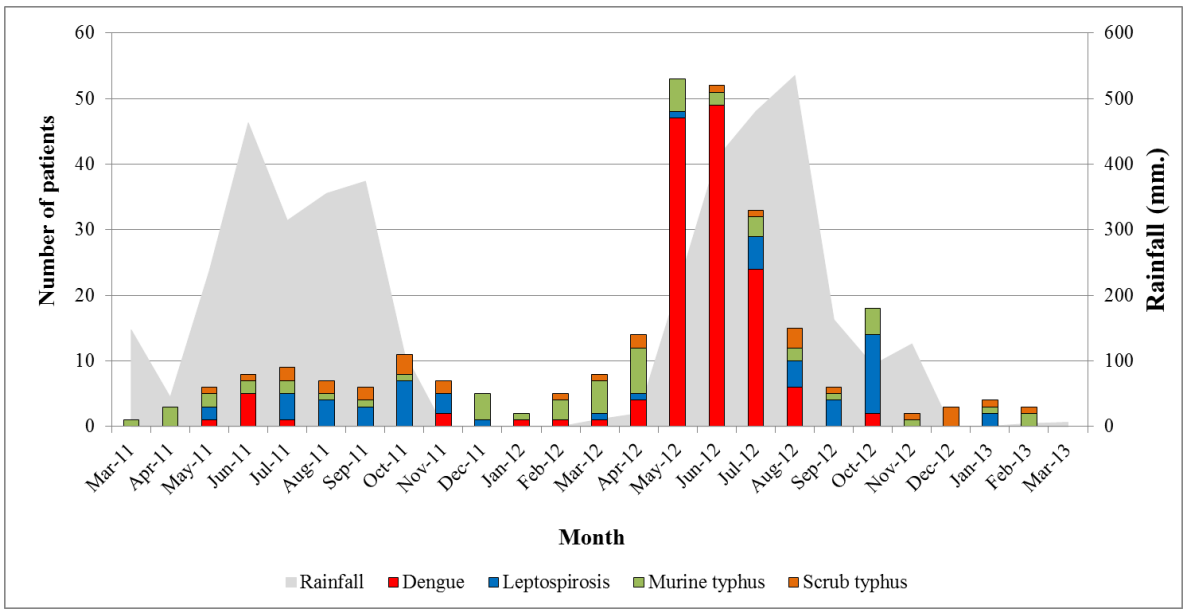


Figure 4.4 The seasonality of dengue, leptospirosis, murine typhus and scrub typhus between March 2011 and March 2013 by month.

(Rainfall data provided by Mae Sot Meteorology Station, Thai Meteorological Department).

4.3.4 Demographics, clinical presentations and laboratory findings of dengue and non-dengue cases

Of the 908 non-malaria febrile patients, 812 patients had available paired acute and convalescent serum specimens tested and were included in the current analysis. Of these, 17.7% (144/812) of patients were given a laboratory confirmed diagnosis of acute dengue infection by paired serology using the AFRIMS IgM/IgG ELISA assays. Of the 144 confirmed cases, 8.3% (12/144) had acute primary infection and 91.7% (132/144) had acute secondary infection.

The distribution of demographics, clinical presentations and laboratory findings for dengue and non-dengue cases are shown in Table 4.3. Of the 144 patients with confirmed dengue, 45.1% (65/144) were male. The median age of the patients was 15 years (IQR 12-21). The majority of patients, 82.6% (119/144) were from Mae La refugee camp. The

median duration of fever at presentation was two days (IQR 2-3) and the median temperature was 38.7°C (IQR 38.3-39.1). Headache was the most frequent symptom reported (92.4%), followed by red eyes (56.3%), joint pain (55.6%), palpable lymph nodes (47.2%), and muscle pain (43.1 %). Jaundice and abnormal bleeding were uncommon (0.7%).

Table 4.3 Demographics, clinical presentations and laboratory findings of dengue and non-dengue cases (n=812).

Demographics	Dengue cases (n=144)			Non-dengue cases (n=668)		
	Number of patients (%)	Median (IQR)	Median days at presentation (IQR)	Number of patients (%)	Median (IQR)	Median days at presentation (IQR)
Age (years)	-	15.0 (12.0-21.0)	-	-	19.0 (11.5-31.0)	-
Sex: male	65 (45.1)	-	-	430 (64.4)	-	-
Children	84 (58.3)	-	-	300 (44.9)	-	-
Location: refugee clinic	119 (82.6)	-	-	362 (54.2)	-	-
Clinical presentations						
Heart rate (beats/minute)	-	99 (88-105)	-	-	96 (84-104)	-
Respiratory rate (breaths/minute)	-	26 (24-28)	-	-	26 (24-28)	-
Presenting temperature (°C)	-	38.7 (38.3-39.1)	-	-	38.5 (38.2-39.0)	-
Capillary refill time (second)	-	2 (2-2)[a]	-	-	2 (1-2)[b]	-
Blood pressure (mmHg)						
• Systolic	138 (95.8)	100 (90-110)	-	589 (88.2)	100 (100-110)	-
• Diastolic	138 (95.8)	70 (60-70)	-	589 (88.2)	70 (60-70)	-
Fever	144 (100)	-	2 (2-3)	668 (100)	-	2 (2-3)
Headache	133 (92.4)	-	2 (2-3)	605 (90.6)	-	2 (2-3)
Red eyes	81 (56.3)	-	2 (1-2)	175 (26.2)	-	2 (1-2)
Joint pain	80 (55.6)	-	2 (2-3)	368 (55.1)	-	2 (2-3)
Lymph nodes palpable	68 (47.2)	-	N/A	155 (23.2)	-	N/A
Muscle pain	62 (43.1)	-	2 (2-3)	287 (43.0)	-	2 (2-3)
Constipation	30 (20.8)	-	2 (2-3)	110 (16.5)	-	2 (2-3)
Pain behind eyes	23 (16.0)	-	2 (2-3)	99 (14.8)	-	2 (2-3)
Abdominal pain	22 (15.3)	-	2.5 (2-3)	157 (23.5)	-	2 (2-3)
Cough	21 (14.6)	-	3 (2-4)	310 (46.4)	-	2 (2-3)

Table 4.3 Demographics, clinical presentations and laboratory findings of dengue and non-dengue cases (n=812) (continued).

Clinical presentations	Dengue cases (n=144)			Non-dengue cases (n=668)		
	Number of patients (%)	Median (IQR)	Median days at presentation (IQR)	Number of patients (%)	Median (IQR)	Median days at presentation (IQR)
Skin rash	14 (9.7)	-	2 (1-3)	17 (2.5)	-	3 (1-7)
• Petechiae	13 (92.9)	-	2 (1-3)	6 (35.3)	-	1 (1-1)
• Eschar	1 (7.1)	-	3 (3-3)	1 (5.9)	-	7 (7-7)
• Maculopapular rash	-	-	-	1 (5.9)	-	4 (4-4)
• Other types of rash	-	-	-	9 (52.9)	-	7 (3-7)
Stiff neck	12 (8.3)	-	2 (2-3)	40 (6.0)	-	3 (2-3)
Jaundice	1 (0.7)	-	2 (2-2)	17 (2.5)	-	2 (2-4)
Abnormal bleeding	1 (0.7)	-	4 (4-4)	8 (1.2)	-	1 (1-2.5)
Laboratory findings						
White blood cells (10^3/μl)	-	4.7 (3.6-6.1)	-	-	7.9 (6.0-10.8)[c]	-
• Neutrophils (10^3/μl)	-	3.1 (2.5-4.7)	-	-	5.7 (4.1-8.3)[d]	-
• Lymphocytes (10^3/μl)	-	0.6 (0.4-0.7)	-	-	1.0 (0.7-1.4)[b]	-
Haematocrit (%)	-	40.1 (37.9-43.2)	-	-	40.0 (37.1-43.2)[c]	-
Platelets (10^3/μl)	-	195.5 (155.5-241)	-	-	222 (170-272)[c]	-
C-reactive protein (mg/l)	-	9.0 (7.9-18.0)	-	-	25.3 (9.0-72.0)[c]	-

[a]Data available in 143/144 (99.3%) cases, [b]Data available in 666/668 (99.7%) cases, [c]Data available in 667/668 (99.9%) cases, [d]Data available in 665/668 (99.6%) cases. Non-dengue cases included 54 murine typhus cases, 44 leptospirosis cases, 29 scrub typhus cases, 14 concomitant leptospirosis and scrub typhus cases, 10 invasive bacterial infection cases, 6 JE cases, one concomitant *Streptocuccos pneumoniae* bacteraemia and scrub typhus case, and 510 unknown fever cases

4.3.5 Clinical characteristics associated with acute dengue infection

Acute dengue infection was significantly associated with age (negative affect), red eyes, cough (negative affect), skin rash, neutrophils (negative affect), lymphocytes (negative affect), and CRP concentration (negative affect) when both univariate and multivariate logistic regression analysis were performed (Table 4.4 and Table 4.5). Sex, location, presenting temperature, days of having red eyes, days of having a cough, abdominal pain, palpable lymph node, and platelets were significantly associated with acute dengue infection when univariate analysis was performed, but did not remain significantly associated when multivariate logistic regression was performed (Table 4.4).

The risk of being diagnosed with acute dengue infection was increased by 3.1 times if a patient had a skin rash and 2.8 times risk if they had red eyes. The risk of being diagnosed with acute dengue infection was reduced by 97.5% for every one unit (10^3/µl) increment of lymphocytes, 85.4% if the patient had a cough, 38.9% for every one unit (10^3/µl) increment of neutrophils, 7.4% for every one year of age increment, and 2.6% for every one unit (mg/l) increment of CRP concentration.

Table 4.4 Univariate logistic regression results for acute dengue infection.

Variables	Odds Ratio (OR)	95% CI	*P*-value
Age (years)	**0.971**	**0.955-0.987**	**<0.001**
Sex: male	**0.455**	**0.316-0.656**	**<0.001**
Location: refugee clinic	**4.024**	**2.547-6.356**	**<0.001**
Presenting temperature (°C)	**1.416**	**1.032-1.942**	**0.031**
Days of fever	0.886	0.760-1.034	0.124
Headache	1.259	0.646-2.454	0.499
Days of headache	0.973	0.852-1.112	0.691
Stiff neck	1.427	0.729-2.794	0.299
Days of stiff neck	1.043	0.831-1.310	0.715
Pain behind the eyes	1.092	0.666-1.791	0.726
Days of pain behind the eyes	1.000	0.842-1.187	0.997
Red eyes	**3.622**	**2.498-5.253**	**<0.001**
Days of red eyes	**1.442**	**1.251-1.661**	**<0.001**
Jaundice	0.268	0.035-2.029	0.202
Days of jaundice	0.572	0.228-1.438	0.235
Muscle pain	1.004	0.698-1.444	0.984
Days of muscle pain	0.972	0.864-1.092	0.630
Joint pain	1.019	0.709-1.464	0.919
Days of joint pain	0.966	0.862-1.083	0.558
Cough	**0.197**	**0.121-0.321**	**<0.001**
Days of cough	**0.606**	**0.503-0.729**	**<0.001**
Constipation	1.335	0.850-2.096	0.209
Days of constipation	1.063	0.904-1.249	0.461
Abdominal pain	**0.587**	**0.360-0.956**	**0.032**
Days of abdominal pain	0.849	0.713-1.011	0.066
Abnormal bleeding	0.577	0.072-4.649	0.605
Days of abnormal bleeding	1.043	0.584-1.864	0.887
Skin rash	**4.124**	**1.983-8.575**	**<0.001**
Days of skin rash	1.032	0.920-1.157	0.594
Lymph nodes palpable	**2.961**	**2.039-4.301**	**<0.001**
White blood cells (10^3/µl)	**0.632**	**0.574-0.695**	**<0.001**
Neutrophils (10^3/µl)	**0.635**	**0.572-0.704**	**<0.001**
Lymphocytes (10^3/µl)	**0.029**	**0.014-0.059**	**<0.001**
Haematocrit (%)	1.002	0.970-1.036	0.895
Platelets (10^3/µl)	**0.996**	**0.994-0.998**	**0.001**
C-reactive protein (mg/l)	**0.969**	**0.959-0.979**	**<0.001**

CI= confidence intervals

Table 4.5 Multivariate logistic regression results for acute dengue infection.

Variables	Odds Ratio (OR)	95% CI	*P*-value
Age (years)	0.926	0.901-0.953	<0.001
Red eyes	2.805	1.649-4.771	<0.001
Cough	0.146	0.079-0.271	<0.001
Skin rash	3.139	1.153-8.548	0.025
Neutrophils (10^3/μl)	0.611	0.525-0.712	<0.001
Lymphocytes (10^3/μl)	0.025	0.010-0.062	<0.001
C-reactive protein (mg/l)	0.974	0.963-0.985	<0.001

CI= confidence intervals

4.3.6 Demographics, clinical presentations and laboratory findings of leptospirosis and non-leptospirosis cases

Acute plasma specimens from all 908 non-malaria febrile patients were tested by 16S *rRNA* qPCR assay for the detection of *Leptospira* DNA. The *Leptospira* DNA was detected in 68 patients. Of these, 14 patients had concomitant leptospirosis and scrub typhus infection proven by the16S *rRNA* qPCR assay for leptospirosis and the reference paired serology for scrub typhus.

Of the 908 patients tested, 894 patients were included in the current analysis (14 patients with leptospirosis and scrub typhus co-infection were excluded). Of these, 54 patients were defined as having acute leptospirosis infection and 840 as not having acute leptospirosis infection.

The patient demographics, clinical presentations and laboratory findings for leptospirosis and non-leptospirosis cases are shown in Table 4.6. Of the 54 patients with confirmed leptospirosis, 79.6% (43/54) were male. The median age of the patients was 15 years (IQR 11-23), and 74.1% (40/54) of the patients were from Mae La refugee camp. The follow-up rate was 81.5% (44/54). The median duration of fever at presentation was two days (IQR 2-3) and the median temperature was 38.8°C (IQR 38.3-39.2). Headache

was the most frequent symptom reported (94.4%), followed by muscle pain (55.6%), joint pain (53.7%), and red eyes (42.6%). Abnormal bleeding was uncommon (1.9%) and no patient reported having skin rash.

Table 4.6 Demographics, clinical presentations and laboratory findings of leptospirosis and non-leptospirosis cases (n=894, excluded 14 patients with leptospirosis and scrub typhus co-infection).

Demographics	Leptospirosis cases (n=54)			Non-leptospirosis cases (n=840)		
	Number of patients (%)	Median (IQR)	Median days at presentation (IQR)	Number of patients (%)	Median (IQR)	Median days at presentation (IQR)
Age (years)	-	15 (11-23)	-	-	19 (12-30)	-
Sex: male	43 (79.6)	-	-	507 (60.4)	-	-
Children	30 (55.6)	-	-	374 (44.5)	-	-
Location: refugee clinic	40 (74.1)	-	-	459 (54.6)	-	-
Clinical presentations						
Heart rate (beats/minute)	-	99 (90-108)	-	-	96 (84-104)	-
Respiratory rate (breaths/minute)	-	26 (24-29)	-	-	26 (24-28)	-
Presenting temperature (°C)	-	38.8 (38.3-39.2)	-	-	38.5 (38.2-39.0)	-
Capillary refill time (second)	-	2 (2-2)[a]	-	-	2 (1-2)[b]	-
Blood pressure (mmHg)						
• Systolic	51 (94.4)	100 (90-110)	-	750 (89.3)	100 (100-110)	-
• Diastolic	51 (94.4)	70 (60-70)	-	750 (89.3)	70 (60-70)	-
Fever	54 (100)	-	2 (2-3)	840 (100)	-	2 (2-3)
Headache	51 (94.4)	-	2 (2-3)	762 (90.7)	-	2 (2-3)
Muscle pain	30 (55.6)	-	2 (2-3)	353 (42.0)	-	2 (2-3)
Joint pain	29 (53.7)	-	2 (2-3)	466 (55.5)	-	2 (2-3)
Red eyes	23 (42.6)	-	2 (1-2)	248 (29.5)	-	2 (1-2)
Abdominal pain	14 (25.9)	-	2 (1-2)	181 (21.5)	-	2 (2-3)
Cough	13 (24.1)	-	2 (2-3)	351 (41.8)	-	2 (2-3)
Lymph nodes palpable	13 (24.1)	-	N/A	221 (26.3)	-	N/A
Constipation	8 (14.8)	-	2 (2-2.5)	149 (17.7)	-	2 (2-3)

Table 4.6 Demographics, clinical presentations and laboratory findings of leptospirosis and non-leptospirosis cases (n=894, excluded 14 patients with leptospirosis and scrub typhus co-infection) (continued).

Clinical presentations	Leptospirosis cases (n=54)			Non-leptospirosis cases (n=840)		
	Number of patients (%)	Median (IQR)	Median days at presentation (IQR)	Number of patients (%)	Median (IQR)	Median days at presentation (IQR)
Pain behind eyes	7 (13.0)	-	2 (2-3)	121 (14.4)	-	2 (2-3)
Stiff neck	5 (9.3)	-	2 (2-2)	53 (6.3)	-	3 (2-3)
Jaundice	4 (7.4)	-	3 (2.5-3.5)	18 (2.1)	-	2 (1-4)
Abnormal bleeding	1 (1.9)	-	1 (1-1)	8 (1.0)	-	1.5 (1-3.5)
Skin rash	0 (0)	-	-	32 (3.8)	-	2 (1-4)
• Petechiae	-	-	-	19 (59.4)	-	2 (1-2)
• Eschar	-	-	-	2 (6.3)	-	5 (3-7)
• Maculopapular rash	-	-	-	1 (3.1)	-	4 (4-4)
• Other types of rash	-	-	-	10 (31.3)	-	6 (2-7)
Laboratory findings						
White blood cells (10^3/μl)	-	9.5 (7.5-13.0)	-	-	6.9 (5.1-9.7)[c]	-
• Neutrophils (10^3/μl)	-	8.2 (6.2-10.6)[a]	-	-	5.0 (3.4-7.3)[b]	-
• Lymphocytes (10^3/μl)	-	0.7 (0.5-0.9)[a]	-	-	1.0 (0.6-1.4)[d]	-
Haematocrit (%)	-	39.3 (37.1-42.0)	-	-	40.2 (37.2-43.4)[c]	-
Platelets (10^3/μl)	-	196 (145-230)	-	-	216 (168-270)[c]	-
C-reactive protein (mg/l)	-	106.5 (40.8-166.5)	-	-	18.0 (7.9-50.6)[c]	-

[a]Data available in 53/54 (98.1%) cases, [b]Data available in 837/840 (99.6%) cases, [c]Data available in 839/840 (99.9%) cases, [d]Data available in 838/840 (99.8%) cases. Non-leptospirosis cases included 144 dengue cases, 54 murine typhus cases, 29 scrub typhus cases, 11 invasive bacterial infection cases, 6 JE cases, one concomitant *Streptococcus pneumoniae* bacteraemia and scrub typhus case, and 595 unknown fever cases

4.3.7 Clinical characteristics associated with acute leptospirosis infection

Acute leptospirosis infection was significantly associated with age (negative effect), location, days of fever (negative effect), days of cough (negative effect), platelets (negative effect), and CRP concentration when both univariate and multivariate logistic regression analysis were performed (Table 4.7 and Table 4.8). Sex, presenting temperature, red eyes, jaundice and days of jaundice, cough, WBC and its differential counts including neutrophils and lymphocytes were significantly associated with acute leptospirosis infection when univariate analysis was performed, but did not remain significantly associated when multivariate logistic regression was performed (Table 4.7).

The risk of being diagnosed with acute leptospirosis infection was increased by 2.7 times if a patient was from the refugee clinic and 2.2% for every one unit (mg/ml) increment of CRP concentration. The risk of being diagnosed with acute leptospirosis infection was reduced by 40.6% for every one day increment of duration of fever, 33.9% for every one day increment of duration of cough, 6.9% for every one year of age increment, and 0.8% for every one unit ($10^3/\mu l$) increment of platelets.

Table 4.7 Univariate logistic regression results for acute leptospirosis infection.

Variables	Odds Ratio (OR)	95% CI	*P*-value
Age (years)	**0.972**	**0.948-0.997**	**0.030**
Sex: male	**2.568**	**1.305-5.050**	**0.006**
Location: refugee clinic	**2.372**	**1.271-4.425**	**0.007**
Presenting temperature (°C)	**1.620**	**1.019-2.575**	**0.041**
Days of fever	**0.734**	**0.558-0.964**	**0.026**
Headache	1.740	0.531-5.706	0.361
Days of headache	0.885	0.716-1.095	0.261
Stiff neck	1.515	0.579-3.962	0.397
Days of stiff neck	0.999	0.710-1.406	0.996
Pain behind the eyes	0.885	0.391-2.004	0.769
Days of pain behind the eyes	0.960	0.721-1.277	0.778
Red eyes	**1.771**	**1.012-3.099**	**0.045**
Days of red eyes	1.152	0.930-1.429	0.196
Jaundice	**3.653**	**1.192-11.201**	**0.023**
Days of jaundice	**1.422**	**1.031-1.960**	**0.032**
Muscle pain	1.725	0.991-3.001	0.054
Days of muscle pain	1.038	0.876-1.231	0.665
Joint pain	0.931	0.536-1.617	0.800
Days of joint pain	0.885	0.733-1.070	0.208
Cough	**0.442**	**0.233-0.837**	**0.012**
Days of cough	**0.739**	**0.577-0.946**	**0.016**
Constipation	0.807	0.373-1.744	0.585
Days of constipation	0.895	0.674-1.189	0.444
Abdominal pain	1.274	0.678-2.394	0.451
Days of abdominal pain	0.911	0.715-1.160	0.449
Abnormal bleeding	1.962	0.241-15.981	0.529
Days of abnormal bleeding	0.943	0.311-2.861	0.917
Lymph nodes	0.888	0.467-1.688	0.717
White blood cells (10^3/µl)	**1.118**	**1.062-1.176**	**<0.001**
Neutrophils (10^3/µl)	**1.171**	**1.104-1.241**	**<0.001**
Lymphocytes (10^3/µl)	**0.157**	**0.071-0.344**	**<0.001**
Haematocrit (%)	0.967	0.918-1.019	0.208
Platelets (10^3/µl)	**0.994**	**0.990-0.998**	**0.002**
C-reactive protein (mg/l)	**1.016**	**1.012-1.020**	**<0.001**

CI= confidence intervals

Table 4.8 Multivariate logistic regression results for acute leptospirosis infection.

Variables	Odds Ratio (OR)	95% CI	*P*-value
Age (years)	0.931	0.898-0.964	<0.001
Location: refugee clinic	2.686	1.312-5.497	0.007
Days of fever	0.594	0.430-0.819	0.002
Days of cough	0.661	0.492-0.888	0.006
Platelets (10^3/μl)	0.992	0.987-0.997	0.001
C-reactive protein (mg/l)	1.022	1.017-1.027	<0.001

CI= confidence intervals

4.3.8 Demographics, clinical presentations and laboratory findings of murine typhus and non-murine typhus cases

Acute and convalescent serum specimens were available for 811 patients and were included in the current analysis. Of these, 54 patients were given laboratory confirmed diagnosis of acute murine typhus infection by paired serology using the MORU in-house IgM ELISA and IFA assays.

The distribution of patient demographics, clinical presentations and laboratory findings for murine typhus and non-murine typhus cases are described in Table 4.9. Of the 54 patients with confirmed murine typhus, 55.6% (30/54) were male. The median age of the patients was 26 years (IQR 18-37), and 70.4% (38/54) of the patients were from Mae La refugee camp. The median duration of fever at presentation was three days (IQR 2-4) and the median temperature was 38.5°C (IQR 38.2-39.8). Headache was the most frequent symptom reported (90.7%), followed by joint pain (64.8%) and muscle pain (55.6%). No patients reported having abnormal bleeding and skin rash.

Table 4.9 Demographics, clinical presentations and laboratory findings of murine typhus and non-murine typhus cases (n=811).

Demographics	Murine typhus cases (n=54)			Non-murine typhus cases (n=757)		
	Number of patients (%)	Median (IQR)	Median days at presentation (IQR)	Number of patients (%)	Median (IQR)	Median days at presentation (IQR)
Age (years)	-	26 (18-37)	-	-	18.0 (11.0-28.0)	-
Sex: male	30 (55.6)	-	-	464 (61.3)	-	-
Children	10 (18.5)	-	-	373 (49.3)	-	-
Location: refugee clinic	38 (70.4)	-	-	443 (58.5)	-	-
Clinical presentations						
Heart rate (beats/minute)	-	89 (80-100)	-	-	96 (84-104)	-
Respiratory rate (breaths/minute)	-	24 (22-26)	-	-	26 (24-28)	-
Presenting temperature (°C)	-	38.5 (38.2-38.9)	-	-	38.5 (38.2-39.0)	-
Capillary refill time (second)	-	2 (1-2)	-	-	2 (2-2)[a]	-
Blood pressure (mmHg)						
• Systolic	53 (98.1)	110 (100-110)	-	674 (89.0)	100 (90-110)	-
• Diastolic	53 (98.1)	70 (70-80)	-	674 (89.0)	70 (60-70)	-
Fever	54 (100)	-	3 (2-4)	757 (100)	-	2 (2-3)
Headache	49 (90.7)	-	3 (2-4)	688 (90.9)	-	2 (2-3)
Joint pain	35 (64.8)	-	3 (2-5)	413 (54.6)	-	2 (2-3)
Muscle pain	30 (55.6)	-	3 (2-4)	319 (42.1)	-	2 (2-3)
Cough	20 (37.0)	-	3 (2-4.5)	311 (41.1)	-	2 (2-3)
Lymph nodes palpable	14 (25.9)	-	N/A	209 (27.6)	-	N/A
Pain behind eyes	12 (22.2)	-	3 (2-4.5)	110 (14.5)	-	2 (2-3)
Red eyes	12 (22.2)	-	2 (1-4)	244 (32.2)	-	2 (1-2)
Abdominal pain	11 (20.4)	-	2 (2-4)	168 (22.2)	-	2 (2-3)
Constipation	7 (13.0)	-	2 (2-4)	133 (17.6)	-	2 (2-3)

Table 4.9 Demographics, clinical presentations and laboratory findings of murine typhus and non-murine typhus cases (n=811) (continued).

Clinical presentations	Murine typhus cases (n=54)			Non-murine typhus cases (n=757)		
	Number of patients (%)	Median (IQR)	Median days at presentation (IQR)	Number of patients (%)	Median (IQR)	Median days at presentation (IQR)
Jaundice	3 (5.6)	-	2 (1-6)	15 (2.0)	-	2 (2-4)
Stiff neck	2 (3.7)	-	5 (3-7)	50 (6.6)	-	2 (2-3)
Abnormal bleeding	0 (0)	-	-	9 (1.2)	-	1 (1-3)
Skin rash	0 (0)	-	-	31 (4.1)	-	2 (1-4)
• Petechiae	-	-	-	19 (61.3)	-	2 (1-2)
• Eschar	-	-	-	2 (6.5)	-	5 (3-7)
• Maculopapular rash	-	-	-	1 (3.2)	-	4 (4-4)
• Other types of rash	-	-	-	9 (29.0)	-	7 (3-7)
Laboratory findings						
White blood cells (10^3/μl)	-	5.6 (3.9-7.5)	-	-	7.4 (5.3-10.3)[b]	-
• Neutrophils (10^3/μl)	-	3.8 (2.6-5.2)	-	-	5.5 (3.7-7.8)[a]	-
• Lymphocytes (10^3/μl)	-	1.0 (0.7-1.6)	-	-	0.9 (0.6-1.3)[c]	-
Haematocrit (%)	-	39.3 (36.9-43.0)	-	-	40.1 (37.3-43.2)[b]	-
Platelets (10^3/μl)	-	196.5 (150-238)	-	-	217 (170-269)[b]	-
C-reactive protein (mg/l)	-	24.7 (15.0-48.6)	-	-	19.0 (7.9-61.0)[b]	-

[a]Data available in 754/757 (99.6%) cases, [b]Data available in 756/757 (99.9%) cases, [c]Data available in 755/757 (99.7%) cases

Non-murine typhus cases included 144 dengue cases, 44 leptospirosis cases, 29 scrub typhus cases, 14 concomitant leptospirosis and scrub typhus cases, 10 invasive bacterial infection cases, 6 JE cases, one concomitant *S. pneumoniae* bacteraemia and scrub typhus case, and 509 unknown fever cases

4.3.9 Clinical characteristics associated with acute murine typhus infection

Acute murine typhus infection was significantly associated with age, days of fever and neutrophils (negative effect) when both univariate and multivariate logistic regression analysis were performed (Table 4.10 and Table 4.11). Days of headache, days of pain behind the eyes, days of muscle pain, and days of joint pain were significantly associated with acute murine typhus infection when univariate analysis was performed, but did not remain significantly associated when multivariate logistic regression was performed (Table 4.10). The risk of being diagnosed with acute murine typhus was increased by 1.5 times for every one day increment of duration of fever and 3.4% for every one year of age increment. The risk of being diagnosed with acute murine typhus infection was reduced by 17.8% for every one unit (10^3/µl) increment of neutrophils.

Table 4.10 Univariate logistic regression results for acute murine typhus infection.

Variables	Odds Ratio (OR)	95% CI	*P*-value
Age (years)	**1.035**	**1.015-1.056**	**<0.001**
Sex: male	0.789	0.453-1.377	0.405
Location: refugee clinic	1.683	0.922-3.073	0.090
Presenting temperature (°C)	0.837	0.493-1.420	0.509
Days of fever	**1.550**	**1.304-1.844**	**<0.001**
Headache	0.983	0.379-2.549	0.972
Days of headache	**1.388**	**1.168-1.649**	**<0.001**
Stiff neck	0.544	0.129-2.298	0.407
Days of stiff neck	1.021	0.716-1.455	0.909
Pain behind the eyes	1.681	0.858-3.293	0.130
Days of pain behind the eyes	**1.275**	**1.051-1.548**	**0.014**
Red eyes	0.601	0.311-1.162	0.130
Days of red eyes	0.969	0.751-1.249	0.805
Jaundice	2.910	0.816-10.378	0.100
Days of jaundice	1.329	0.928-1.905	0.121
Muscle pain	1.716	0.984-2.992	0.057
Days of muscle pain	**1.302**	**1.124-1.507**	**<0.001**
Joint pain	1.534	0.862-2.731	0.146
Days of joint pain	**1.302**	**1.122-1.512**	**0.001**
Cough	0.844	0.477-1.493	0.559
Days of cough	1.078	0.915-1.270	0.369
Constipation	0.699	0.309-1.580	0.389
Days of constipation	0.929	0.698-1.235	0.610
Abdominal pain	0.897	0.453-1.778	0.755
Days of abdominal pain	1.027	0.835-1.265	0.799
Lymph nodes	0.918	0.489-1.722	0.789
White blood cells (10^3/µl)	**0.847**	**0.768-0.933**	**0.001**
Neutrophils (10^3/µl)	**0.792**	**0.700-0.895**	**<0.001**
Lymphocytes (10^3/µl)	1.226	0.851-1.765	0.274
Haematocrit (%)	0.978	0.928-1.030	0.402
Platelets (10^3/µl)	0.997	0.993-1.000	0.076
C-reactive protein (mg/l)	0.997	0.992-1.003	0.367

CI= confidence intervals

Table 4.11 Multivariate logistic regression results for acute murine typhus infection.

Variables	Odds Ratio (OR)	95% CI	*P*-value
Age (years)	1.034	1.012-1.056	0.002
Days of fever	1.552	1.291-1.866	<0.001
Neutrophils (10^3/μl)	0.822	0.730-0.926	0.001

CI= confidence intervals

4.3.10 Demographics, clinical presentations and laboratory findings of scrub typhus and non-scrub typhus cases

Acute and convalescent serum specimens were available for 811 patients and were tested by paired serology using the MORU in-house IgM ELISA and IFA assays for scrub typhus. Of these, 44 patients were given a laboratory confirmed diagnosis of acute scrub typhus infection. Of the 44 patients, 14 patients had concomitant leptospirosis and scrub typhus infection proven by the 16S *rRNA* qPCR assay (leptospirosis) and the reference paired serology (scrub typhus). One patient had concomitant *S. pneumoniae* bacteraemia and scrub typhus proven by blood culture (*S. pneumoniae*) and the reference paired serology (scrub typhus).

Of the 811 patients, 796 patients were included in the current analysis (excluding 15 patients with co-infections). Of these, 29 patients were defined as having acute scrub typhus infection and 767 patients as not having acute scrub typhus infection.

The distribution of patient demographics, clinical presentations and laboratory findings for scrub typhus and non-scrub typhus cases are shown in Table 4.12. Of the 29 patients with confirmed scrub typhus, 69.0% (20/29) were male. The median aged of the patients was 18 years (IQR 12-33), and 58.6% (17/29) were from the refugee clinic. The median duration of fever at presentation was three days (IQR 2-4) and the median temperature was 38.5°C (IQR 38.3-39.0). Headache was the most frequent symptom

reported (89.7%), followed by joint pain (51.7%). No patients reported having abnormal bleeding and skin rash.

Table 4.12 Demographics, clinical presentations and laboratory findings of scrub typhus and non-scrub typhus cases (n=796, excluded 15 patients with co-infections).

Demographics	Scrub typhus cases (n=29)			Non-scrub typhus cases (n=767)		
	Number of patients (%)	Median (IQR)	Median days at presentation (IQR)	Number of patients (%)	Median (IQR)	Median days at presentation (IQR)
Age (years)	-	18 (12-33)	-	-	18 (12-29)	-
Sex: male	20 (69.0)	-	-	463 (60.4)	-	-
Children	14 (48.3)	-	-	362 (47.2)	-	-
Location: refugee clinic	17 (58.6)	-	-	457 (59.6)	-	-
Clinical presentations						
Heart rate (beats/minute)	-	90 (84-104)	-	-	96 (84-104)	-
Respiratory rate (breaths/minute)	-	28 (24-32)	-	-	26 (24-28)	-
Presenting temperature (°C)	-	38.5 (38.3-39.0)	-	-	38.5 (38.2-39.0)	-
Capillary refill time (second)	-	2 (2-2)	-	-	2 (1-2)[a]	-
Blood pressure (mmHg)						
• Systolic	27 (93.1)	100 (90-120)	-	686 (89.4)	100 (100-110)	-
• Diastolic	27 (93.1)	70 (60-70)	-	686 (89.4)	70 (60-70)	-
Fever	29 (100)	-	3 (2-4)	767 (100)	-	2 (2-3)
Headache	26 (89.7)	-	2 (2-3)	697 (90.9)	-	2 (2-3)
Joint pain	15 (51.7)	-	3 (2-5)	420 (54.8)	-	2 (2-3)
Cough	11 (37.9)	-	3 (2-3)	317 (41.3)	-	2 (2-3)
Abdominal pain	11 (37.9)	-	2 (2-3)	165 (21.5)	-	2 (2-3)
Muscle pain	10 (34.5)	-	3 (3-6)	330 (43.0)	-	2 (2-3)
Red eyes	8 (27.6)	-	2 (2-3)	243 (31.7)	-	2 (1-2)
Pain behind eyes	7 (24.1)	-	3 (2-5)	114 (14.9)	-	2 (2-3)
Constipation	6 (20.7)	-	3 (3-3)	130 (16.9)	-	2 (2-3)

Table 4.12 Demographics, clinical presentations and laboratory findings of scrub typhus and non-scrub typhus cases (n=796, excluded 15 patients with co-infections) (continued).

Demographics	Scrub typhus cases (n=29)			Non-scrub typhus cases (n=767)		
	Number of patients (%)	Median (IQR)	Median days at presentation (IQR)	Number of patients (%)	Median (IQR)	Median days at presentation (IQR)
Lymph nodes palpable	6 (20.7)	-	N/A	214 (27.9)	-	N/A
Stiff neck	4 (13.8)	-	2.5 (1.5-3.0)	46 (6.0)	-	3 (2-3)
Jaundice	2 (6.9)	-	4.5 (2-7)	16 (2.1)	-	2 (2-3.5)
Abnormal bleeding	0 (0)	-	-	9 (1.2)	-	1 (1-3)
Skin rash	0 (0)	-	-	31 (4.0)	-	2 (1-4)
• Petechiae	-	-	-	19 (61.3)	-	2 (1-2)
• Eschar	-	-	-	2 (6.5)	-	5 (3-7)
• Maculopapular rash	-	-	-	1 (3.2)	-	4 (4-4)
• Other types of rash	-	-	-	9 (29.0)	-	7 (3-7)
Laboratory findings						
White blood cell (10^3/μl)	-	7.5 (5.7-8.8)	-	-	7.1 (5.2-9.9)[b]	-
• Neutrophils (10^3/μl)	-	5.6 (3.9-7.0)	-	-	5.2 (3.5-7.6)[c]	-
• Lymphocytes (10^3/μl)	-	0.8 (0.7-1.0)	-	-	0.9 (0.6-1.4)[a]	-
Haematocrit (%)	-	39.1(37.2-43.6)	-	-	40.1 (37.3-43.1)[b]	-
Platelet (10^3/μl)	-	18 (130-254)	-	-	216 (170-266)[b]	-
C-reactive protein (mg/l)	-	55.2 (40.7-114.0)	-	-	18.0 (7.9-55.0)[b]	-

[a]Data available in 765/767 (99.7%) cases, [b]Data available in 766/767 (99.9%) cases, [c]Data available in 741/767 (96.6%) cases

Non-scrub typhus cases included 144 dengue cases, 54 murine typhus cases, 44 leptospirosis cases, 10 invasive bacterial infection cases, 6 JE cases, and 509 unknown fever cases

4.3.11 Clinical characteristics associated with acute scrub typhus infection

Acute scrub typhus infection was significantly associated with days of fever and CRP concentration when both univariate and multivariate logistic regression analysis were performed (Table 4.13 and Table 4.14). Days of pain behind the eyes, days of jaundice, and abdominal pain were significantly associated with acute scrub typhus infection when univariate analysis was performed, but did not remain significantly associated when multivariate logistic regression was performed (Table 4.13). The risk of being diagnosed with acute scrub typhus infection was increased by 1.3 times for every one day increment of duration of fever and 0.8% for every one unit (mg/l) increment of CRP concentration.

Table 4.13 Univariate logistic regression results for acute scrub typhus infection.

Variables	Odds Ratio (OR)	95% CI	*P*-value
Age (years)	1.005	0.977-1.034	0.723
Sex: male	1.459	0.656-3.247	0.355
Location: refugee clinic	0.961	0.453-2.040	0.917
Presenting temperature (°C)	1.471	0.780-2.774	0.233
Days of fever	**1.395**	**1.107-1.758**	**0.005**
Headache	0.870	0.257-2.948	0.824
Days of headache	1.201	0.944-1.528	0.136
Stiff neck	2.508	0.838-7.509	0.100
Days of stiff neck	1.194	0.836-1.705	0.330
Pain behind the eyes	1.823	0.761-4.365	0.178
Days of pain behind the eyes	**1.305**	**1.026-1.659**	**0.030**
Red eyes	0.821	0.359-1.881	0.642
Days of red eyes	1.116	0.829-1.501	0.470
Jaundice	3.477	0.761-15.885	0.108
Days of jaundice	**1.541**	**1.072-2.214**	**0.019**
Muscle pain	0.697	0.320-1.519	0.364
Days of muscle pain	1.097	0.884-1.361	0.402
Joint pain	0.885	0.421-1.859	0.747
Days of joint pain	1.079	0.868-1.341	0.493
Cough	0.868	0.404-1.862	0.715
Days of cough	1.063	0.852-1.326	0.590
Constipation	1.278	0.510-3.201	0.600
Days of constipation	1.128	0.833-1.526	0.436
Abdominal pain	**2.230**	**1.033-4.814**	**0.041**
Days of abdominal pain	1.125	0.886-1.428	0.335
Lymph nodes	0.674	0.271-1.678	0.397
White blood cells ($10^3/\mu l$)	0.968	0.877-1.068	0.517
Neutrophils ($10^3/\mu l$)	0.988	0.888-1.100	0.831
Lymphocytes ($10^3/\mu l$)	0.640	0.318-1.289	0.211
Haematocrit (%)	0.991	0.925-1.062	0.806
Platelets ($10^3/\mu l$)	0.996	0.991-1.001	0.089
C-reactive protein (mg/l)	**1.010**	**1.004-1.015**	**0.001**

CI= confidence intervals

Table 4.14 Multivariate logistic regression results for acute scrub typhus infection.

Variables	Odds Ratio (OR)	95% CI	*P*-value
Days of fever	1.284	1.006-1.638	0.044
C-reactive protein (mg/l)	1.008	1.003-1.014	0.004

CI= confidence intervals

4.3.12 The usefulness of non-specific infection markers in the laboratory confirmed cases of dengue, leptospirosis, murine typhus and scrub typhus

The CBC and CRP tests were performed on acute specimens from all patients as part of the routine laboratory testing at SMRU, haematology laboratory. A total of 281 patients with laboratory confirmed diagnosis of dengue (n=144), leptospirosis (n=54), murine typhus (n=54) and scrub typhus (n=29) were included in the current analysis. The results of CRP test and CBC test including WBC count, haematocrit, and platelet count were compared between the four disease groups. The distribution of WBC count, haematocrit, platelet counts, and CRP concentration by diagnosis groups are shown in Figure 4.5.

The distribution of WBC count was significantly different between the disease groups (*P*=0.0001). Patients with dengue diagnosis had significantly lower WBC counts (4.7 $x10^3$/µl [IQR 3.6-6.1]) compared to patients with the other bacterial diagnoses (leptospirosis, *P*<0.0001; murine typhus, *P*=0.0178; and scrub typhus, *P*<0.0001). Amongst those patients with bacterial diagnoses, patients with leptospirosis had significantly higher WBC counts (9.5 $x10^3$/µl [IQR 7.5-13.0]) compared to patients with scrub typhus (7.5 $x10^3$/µl [IQR 5.7-8.8]) and murine typhus (5.6 $x10^3$/µl [IQR 3.9-7.5]) (*P*=0.0010, and *P*<0.0001, respectively). Patients with murine typhus had significantly lower WBC counts compared to patients with scrub typhus (*P*=0.0216), however, most of these patients had WBC counts within the normal range (Figure 4.5). For WBC differentials, neutrophils and lymphocytes were significantly different between the disease

groups, however, most patients had neutrophils within the normal ranges. Patients with confirmed dengue, leptospirosis and scrub typhus had lymphocytes lower than the normal range (Figure 4.5).

CRP concentrations above 5.0 mg/l were reported in all patients. The differences in CRP concentrations between the four diagnosis groups were statistically significant (P=0.0001). Patients with dengue had significantly lower concentration of CRP (9.0 mg/l [IQR 7.9-18.0]) compared to the other bacterial diagnoses (P<0.0001 for all). Amongst patients with bacterial diagnoses, patients with leptospirosis had higher concentration of CRP (106.5 mg/l [IQR 40.8-166.5]), followed by scrub typhus (55.2 mg/l [IQR 40.7-114.0]) and murine typhus (24.7 mg/l [IQR 15.0-48.6]) (Figure 4.5). Patients with murine typhus had significantly lower CRP concentration than those of patients with leptospirosis (P<0.0001) and scrub typhus (P=0.0004). There was no difference in the CRP concentration between patients with leptospirosis and scrub typhus (P=0.0915).

The differences in the distributions of haematocrit, and platelet count were not statistically significant between the four diagnosis groups and most of them were within the normal ranges (Figure 4.5).

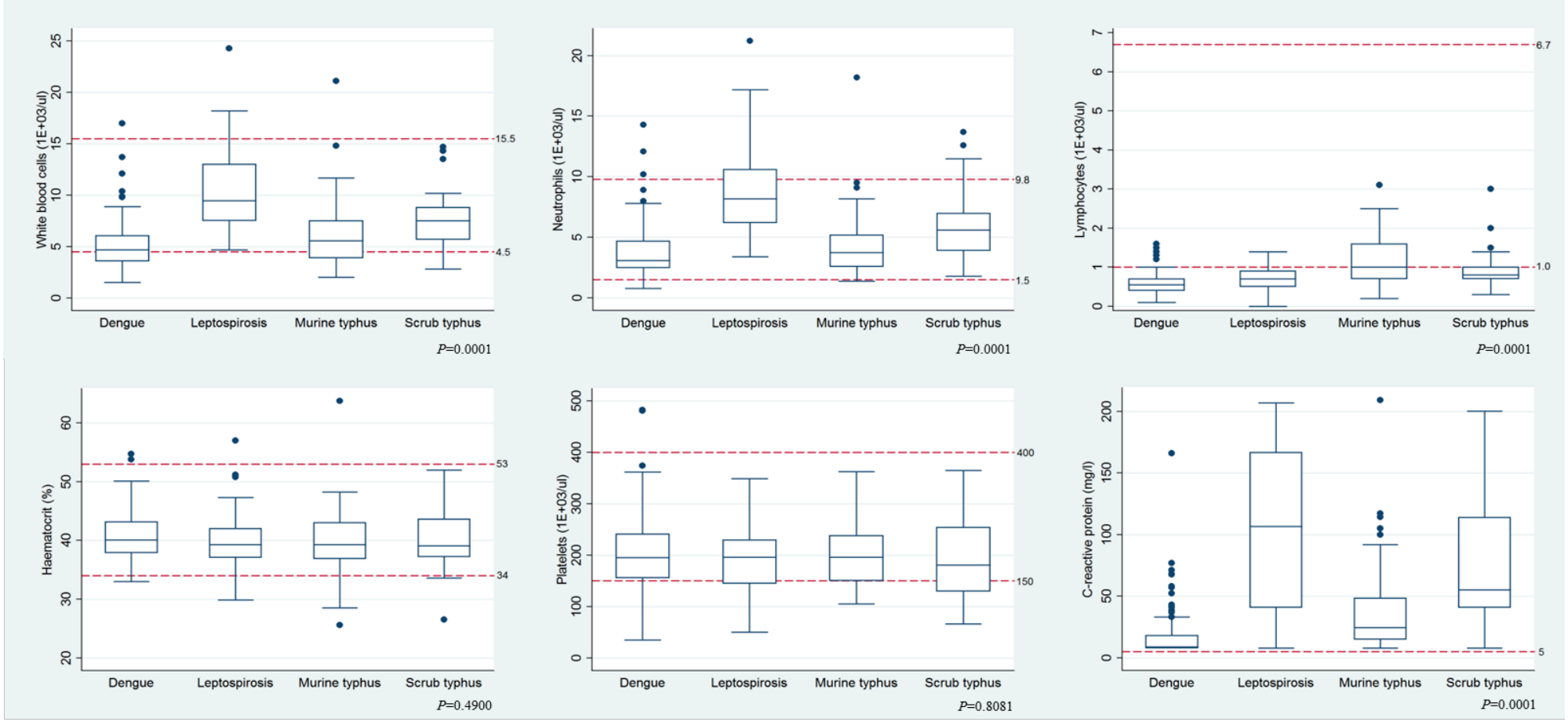


Figure 4.5 Distributions of white blood cells, neutrophils, lymphocytes, haematocrit, platelets and C-reactive protein concentration by diagnosis.

Dashed lines show normal ranges (source: Haematology laboratory, SMRU [272-274] and SMRU CBC data).

4.3.13 Sub-microscopic malaria

Acute packed RBC specimens were available from all 908 patients who were reported malaria negative by microscopy or RDT (Standard Diagnostics) at the clinics. Of these, malaria DNA was detected in 17.5% (159/908) of the patients by the 18S *rRNA* qPCR assay specific for *Plasmodium* sp. The number of patients with the qPCR positive by reference diagnosis is shown in Table 4.15.

Table 4.15 Number of patients with malaria qPCR positive by diagnosis.

Diagnosis	Number of patients (%)
No diagnosis	115 (72.3)
Dengue	18 (11.3)
Leptospirosis	11 (6.9)
Scrub typhus	7 (4.4)
Invasive bacterial infection	3 (1.9)
Leptospirosis/ Scrub typhus	2 (1.3)
Murine typhus	2 (1.3)
S. pneumoniae bacteraemia/ Scrub typhus	1 (0.6)
Total	**159 (100)**

The proportion of malaria qPCR positive in patients that were malaria smear/RDT negative by each diagnosis group is shown in Figure 4.6. Overall, the malaria DNA was detected in almost all diagnosis groups. The proportion of malaria qPCR positive specimens in patients with unknown diagnosis was 19.3% (115/595; 95% CI 16.2-22.7) compared to 14.1% (44/313; CI 10.4-18.4) of patients with a diagnosis (P=0.047).

Amongst the patients with known diagnosis, a positive malaria qPCR was reported in 12.5% (18/144; 95% CI 7.6-19.0) of patients with dengue, 20.4% (11/54; 95% CI 10.6-33.5) of patients with leptospirosis, 24.1% (7/29; 95% CI 10.3-43.5) of patients with scrub typhus, 27.3.0% (3/11; 95% CI 6.0-61.0) of patients with invasive bacterial infection, 3.7% (2/54; 95% CI 0.5-12.7) of patients with murine typhus, 14.3% (2/14; 95% CI 1.8-42.8) of patients with concomitant leptospirosis and scrub typhus, and 100% (1/1) of the concomitant *S. pneumoniae* bacteraemia and scrub typhus. No positive qPCR was reported in patients with JE. Based on these findings it is possible that some of the undiagnosed patients had malaria infections. To investigate this further, sub-microscopic malaria in these groups is analysed in relation to parasitaemia.

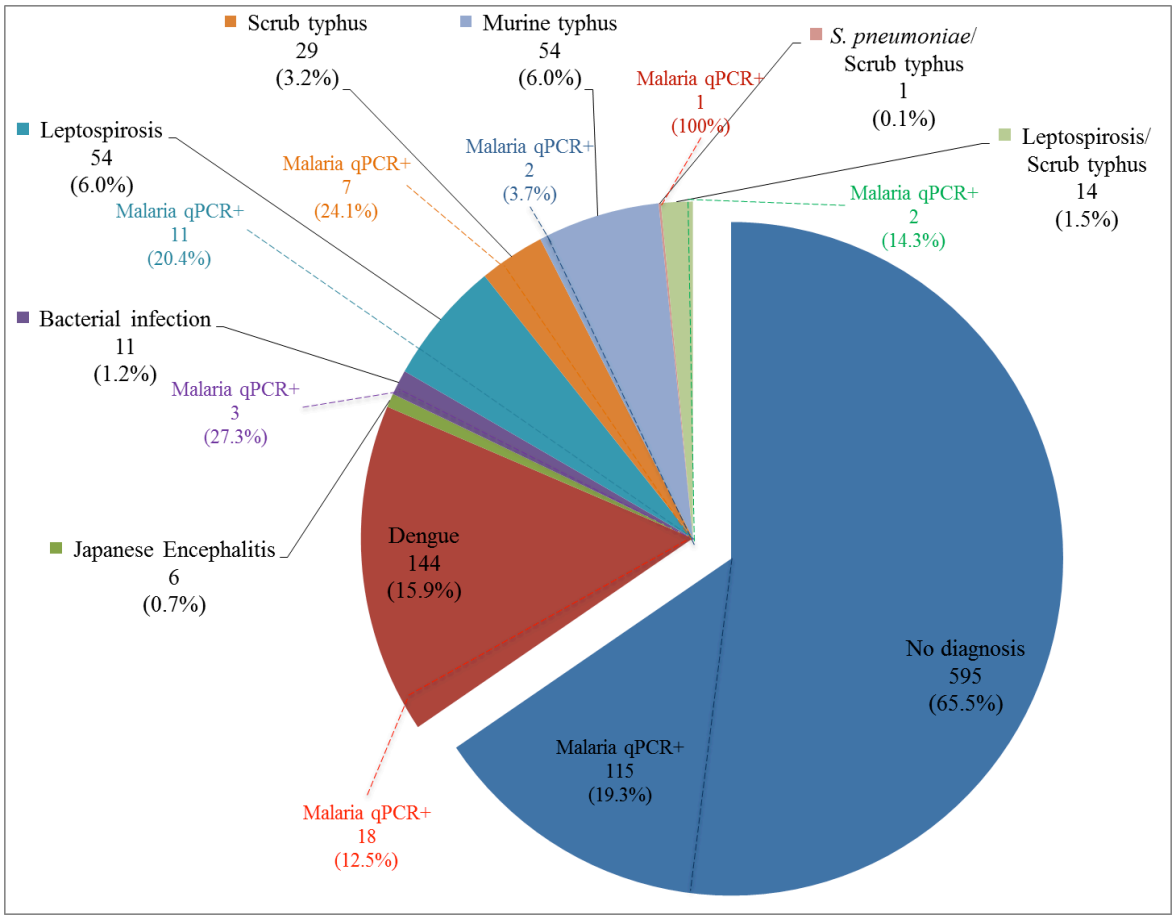


Figure 4.6 Proportion of malaria qPCR positive in patients with smear/RDT negative by diagnosis.

The lower limit of detection (LoD) of this qPCR assay was 12.8 parasites/ml of blood (equivalent to 0.64 parasite/µl of DNA extract). The geometric mean of parasitaemia for 159 positive patients was 281.1 parasites/ml of blood (95% CI 172.5-458.2).

The distribution of malaria parasite density from the 159 qPCR positive patients was analysed by diagnosis groups (Figure 4.7). Overall, the malaria parasitaemia was very low in all diagnosis groups. The geometric mean of malaria parasitaemia in patients with no diagnosis was 312.5 parasites/ml of blood (95% CI 172.0-567.8), a geometric mean of 253.6 parasites/ml (95% CI 75.9-847.5) was reported from patients with dengue, 161.7 parasites/ml (95% CI 15.6-1,670.0) was reported from patients with leptospirosis and 83.4 parasites/ml (95% CI 2.3-3,027.4) was reported from patients with scrub typhus. There were three patients with reported high parasitaemia ($>10^6$ parasites/ml of blood) in the no diagnosis group. The numbers of qPCR positive patients were small in the other diagnosis groups (Figure 4.7). The malaria parasite densities were not statistically different between groups (P=0.9013) and were also not significantly different comparing between patients with diagnosis and the no diagnosis group (P=0.4905) (Figure 4.8).

It is generally accepted that a parasitaemia of more than 5,000-10,000 parasites/ml of blood or 100,000 parasites/ml of blood should be detected by microscopy or RDT, respectively. However, the methods used to calculate parasitaemia by microscopy, RDT, and qPCR are different and therefore it is difficult to compare. For example, the qPCR detects 18S *rRNA* gene of parasite genome where there are 5-8 copies/parasite genome. The detection and calculation are accounted for all stages of malaria parasite including merozoite where it is not accounted in the microscopic examination.

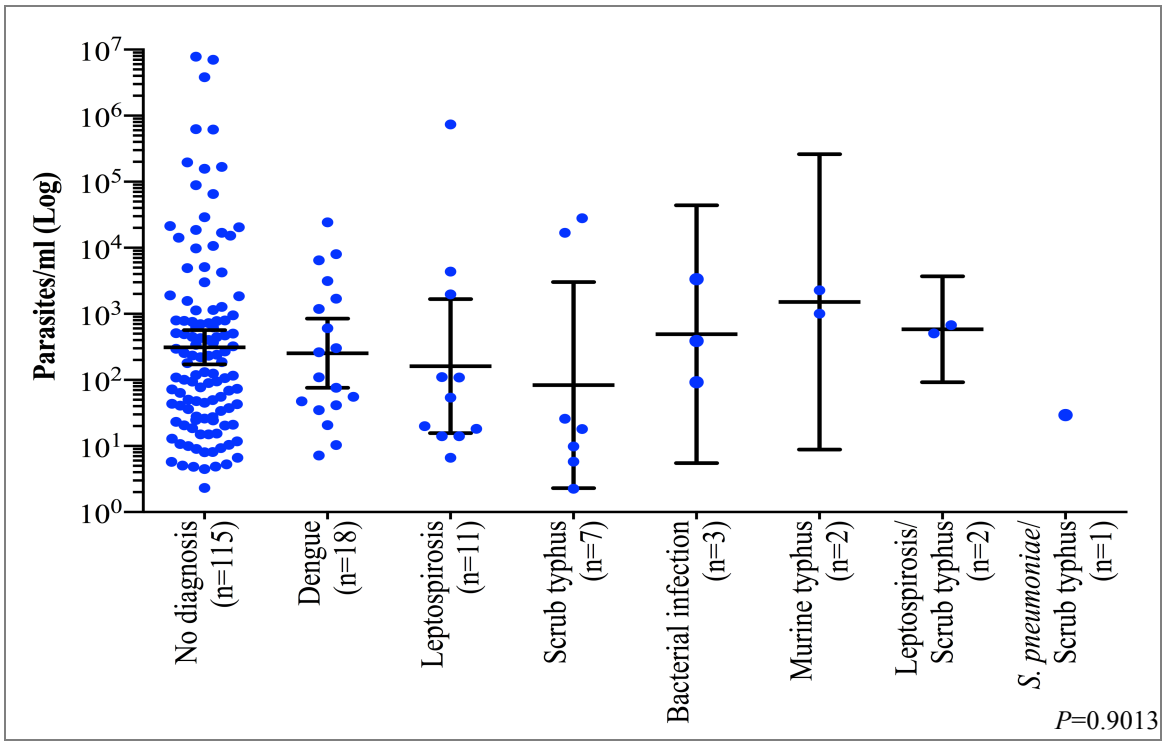


Figure 4.7 Malaria parasitaemia by qPCR assay in patients with smear/RDT negative by diagnosis groups.

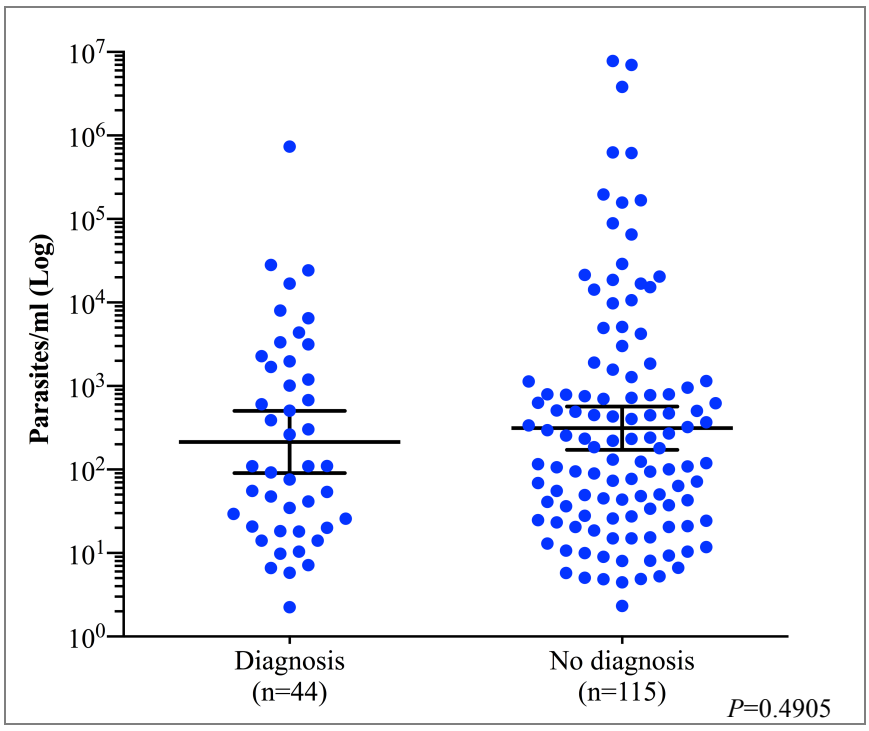


Figure 4.8 Malaria parasitaemia by qPCR assay in patients with smear/RDT negative comparing diagnosis and no diagnosis groups.

DNA extracts from all positive malaria qPCR were tested further for the *P. falciparum* and *P. vivax* speciation by microsatellite nested-PCR assays. The analytical LoD of the assays were five parasites/µl and one parasite/µl for *P. falciparum* and *P. vivax*, respectively. The distribution of malaria qPCR positive patients by species and diagnosis is shown in Table 4.16. Of the 159 patients, 97 patients were found to have *P. vivax*, 22 patients had *P. falciparum*, and 16 patients were detected with mixed *P. falciparum* and *P. vivax*. It was not possible to specify species in the other 24 patients (negative for both *P. falciparum* and *P. vivax*). The geometric mean parasitaemia for *P. vivax*, *P. falciparum*, and mixed *P. falciparum* and *P. vivax* by qPCR were 383.8 parasites/ml of blood (95% CI 223.7-658.3), 344.2 parasites/ml (95% CI 85.8-1,380.7), and 2,514.9 parasites/ml (95% CI 205.8-30,728.9), respectively. The geometric mean for indeterminate species was 15.4 parasites/ml (95% CI 10.1-23.4).

Table 4.16 Distribution of malaria qPCR positive patients by species and diagnosis.

Diagnosis	Number of patients				
	P. falciparum	***P. vivax***	***P. falciparum* and *P. vivax***	**Indeterminate species**	**Total**
No diagnosis	13	74	12	16	115
Dengue	4	10	1	3	18
Leptospirosis	2	5	2	2	11
Scrub typhus	0	4	1	2	7
Invasive bacterial infection	2	1	0	0	3
Murine typhus	1	1	0	0	2
Leptospirosis/ Scrub typhus	0	2	0	0	2
S. pneumoniae bacteraemia/ Scrub typhus	0	0	0	1	1
Total	**22**	**97**	**16**	**24**	**159**

4.4 Results: Malaria patients (PART II)

4.4.1 Malaria and co-infections

In the malaria part of the study (PART II), 121 malaria patients were recruited. All patients were tested and found positive either by malaria smear and/or RDT (SD BIOLINE Malaria Antigen P.f/ Pan POCT, Standard Diagnostics, Inc.) at the enrolment visit (see section 2.5). Of specimens tested by smear only, smear and RDT and RDT only 81, 21 and 19 patients were positive respectively. The distribution of malaria species by smear/RDT at enrolment is shown in Table 4.17. Of the 121 malaria patients, 61 patients were positive for *P. falciparum*, 48 patients were positive for *P. vivax*, and 12 patients were positive for *Plasmodium* other species. The RDT had three lines: "C" (control), "Pan" (*Plasmodium* species; *P. vivax, P. malariae* and *P. ovale*), and "P.f" (*P. falciparum*) lines. According to the SMRU SOP (RT5), a positive "Pan" line was recorded as positive for *P. vivax*. Therefore, in this part of the study, 61 patients were reported having *P. falciparum* and 60 patients were reported having *P. vivax*.

Table 4.17 Distribution of malaria species by smear/RDT at enrolment.

	P. falciparum	***P. vivax***	***Plasmodium* other species**	**Total**
Smear	54	48	NA	102
RDT*	7	NA	12	19
Total	61	48	12	121

*RDT had three lines: control, *P. falciparum*, and *Plasmodium* other species (*P. vivax, P. malariae* and *P. ovale*)

Malaria parasite density was reported from 102 patients who tested positive by smear. The parasite counts were calculated to parasitaemia per ml of blood [(number of parasites counted against 500 WBC x number of WBC/µl of blood x 1,000)/500, or (number of parasites counted against 1,000 RBC x number of RBC/µl of blood x 1,000)/1,000]. The geometric mean parasitaemia was 7.2 $x10^6$ parasites/ml of blood (95% CI 4.9 $x10^6$-1.1 $x10^7$). The malaria qPCR was also performed in this patient group. The geometric mean of 2.2 $x10^6$ parasites/ml of blood (95% CI 1.3 $x10^6$-3.6 $x10^6$) was reported by qPCR.

In addition, the qPCR assay was able to quantify the parasitaemia from 18/19 patients who were positive by the RDT. The geometric mean parasitaemia of the 18 patients was 3.0 $x10^6$ parasites/ml of blood (95% CI 1.7 $x10^6$-5.2 $x10^6$).

From a total of 121 malaria patients, 120 patients were positive by qPCR. The overall parasite density quantified by qPCR was reported with the geometric mean of 2.3 $x10^6$ parasites/ml of blood (95% CI 1.5 $x10^6$-3.5 $x10^6$). This parasite density of the malaria patients (smear/RDT positive) was significantly higher than those of the sub-microscopic malaria patients (2.3 $x10^6$ vs. 281.1 parasites/ml of blood, $P<0.0001$). The qPCR negative patient tested positive by RDT only at the enrolment visit in the clinic.

The malaria species were also identified by the microsatellite nested-PCR assay from all 121 malaria patients (120 patients were qPCR positive). Of these, 55 (45.5%) patients were found to have *P. vivax*, 43 (35.5%) patients had *P. falciparum*, 20 (16.5%) patients were found to have mixed *P. falciparum* and *P. vivax*, and the species was undetermined for three (2.5%) patients (Table 4.18).

Table 4.18 Distribution of malaria species by microsatellite nested-PCR and smear/RDT.

	Species by smear/RDT		
Species by microsatellite nested-PCR	***P. falciparum***	***P. vivax***	**Total (%)**
P. falciparum	43	0	43 (35.5)
P. falciparum* and *P. vivax	16	4	20 (16.5)
P. vivax	0	55	55 (45.5)
Indeterminate species	2	1	3 (2.5)
Total	61	60	121 (100)

All reference laboratory diagnostic tests for the diagnosis of the other non-malaria causes of fever were performed in the malaria patients as well as in the non-malaria patients to assess the impact of dual infections with malaria. Co-infection was found in 8.3% (10/121) of the malaria patients, of which nine (7.4%) patients had laboratory confirmed diagnosis of acute scrub typhus infection and one (0.8%) patient had acute dengue infection (Figure 4.9).

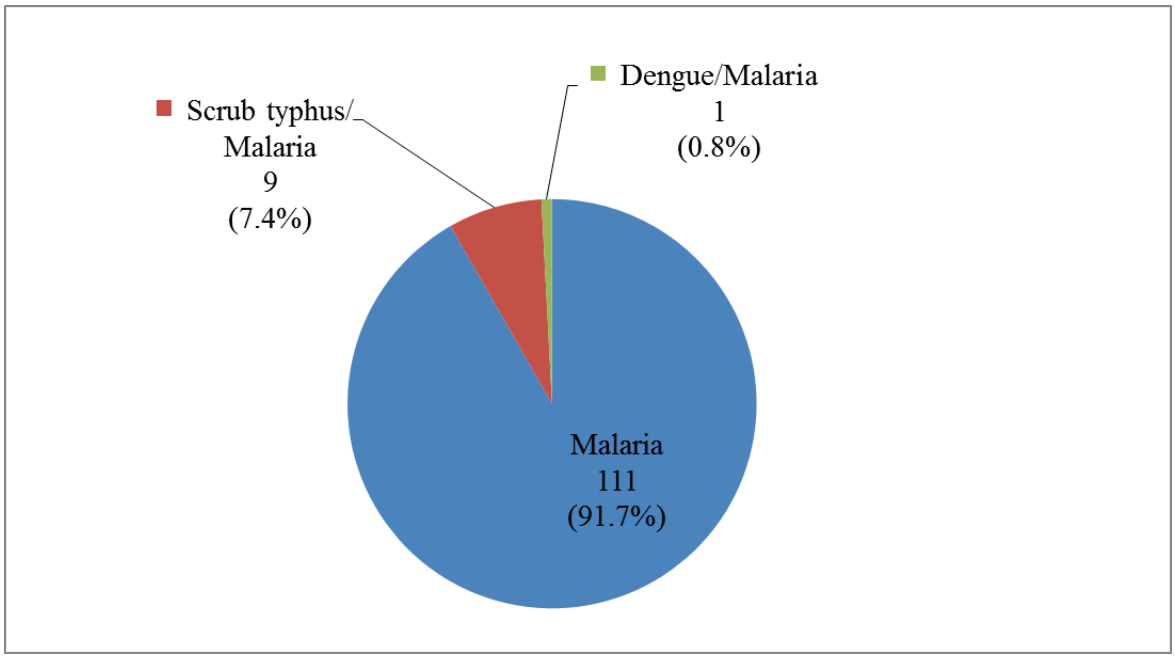


Figure 4.9 Dual infections with 121 malaria febrile patients based on the results of the reference diagnostic tests.

4.5 Discussion

This study aimed to determine the optimal testing strategies and evaluate several diagnostic methodologies for acute diagnosis of the common non-malaria causes of AUFI, including dengue, leptospirosis and rickettsial infections, in a rural and limited resources setting of SE Asia. Although it was not designed as an epidemiological study, the outcome diagnosis from the enrolled patients allowed the study to determine the causes of non-malaria AUFI and estimate the occurrence of these infections in the SMRU clinic populations where the definitive diagnosis of non-malaria AUFI is generally not given and the data are scarce. The causes of non-malaria AUFI were determined for patients presenting to the SMRU clinics over two years of the study period between 2011 and 2013. Dengue, leptospirosis, murine typhus and scrub typhus were found to be common causes of AUFI in this study. This finding has confirmed the results from previously published papers that these infections were common in this region [6, 7, 11, 12, 19, 21]. Using the reference laboratory diagnostic tests, 34.6% of the patients had a confirmed diagnosis in

this study. Previous studies reported rates of laboratory confirmation of diagnoses of between 33.2% and 73.3% (Table 1.1). However, it is difficult to directly compare such studies because of differences in study design and laboratory tests used. There were differences in time of the study, enrolment criteria (history of fever, temperature cut-off points and measurement methods), populations (age, gender, pregnancy status, geographical areas) and very importantly, the laboratory methods and diagnostic algorithms.

Dengue was the most common diagnosis found in the present study, accounting for 15.9% of patients. This was similar to the result of a previous fever study conducted in the same area in 2008, in which dengue was the major cause of AUFI. However, the proportion of dengue cases in that study was almost three times higher than the present study (44.4%) [16]. A study of the causes of febrile illness conducted on the Thailand-Myanmar border between 1999-2002 found that malaria was the most common cause of fever. However, once malaria was excluded, leptospirosis (17.5%) became the most common cause of fever rather than dengue [11]. Leptospirosis and murine typhus were the second most common diagnoses in the present study, accounting for 6.0% of the patients. This was followed by scrub typhus, the third most common, accounting for 3.2%. These studies contrast with the report from an epidemiological study conducted in 10 community-based hospitals in Thailand during 1991-1993 [6]. This study found that scrub typhus was the most common cause of AUFI, accounting for 7.5% and leptospirosis was reported in only 1.1% of cases. Conversely, another study of AUFI conducted between 2001 and 2002 in five hospitals in Thailand reported that leptospirosis was the major cause of adult AUFI accounting for 36.9% of cases, and scrub typhus was the second most common cause found (16.0%) [7]. The number of scrub typhus cases being lower than that of murine typhus in the present study is not consistent with the evidence that scrub typhus is a more rural disease than murine typhus [12, 170], but it is consistent with the previously

published data from SMRU clinics (2004-2006) where a higher number of murine typhus cases was reported [10]. Although, there is no data available for murine typhus in the Thai national reporting system, there is enough evidence from studies and reports that murine typhus is circulating and is common. Scrub typhus and murine typhus coexist in endemic regions [6, 7, 170, 188]. This finding emphasised the need to focus more on murine typhus in the rural areas.

The differences between the present study and previous reports may be explained by several factors. First, there was variation in the study designs, such as inclusion criteria, time of study, geographical areas, age group of patients, pathogens investigated, laboratory diagnostic tests and algorithms used to determine the causes of fever. These differences need to be taken into account when considering different results. For example, in the study of 10 hospitals during 1991-1993 [6], leptospirosis was diagnosed using MAT test that included only *Leptospira bataviae* as an antigen whereas the later study (five hospitals during 2001-2002) used a panel of reference strains from 24 representative serogroups as the antigen in the MAT test and culture for leptospires was also performed [7]. Only qPCR was used to diagnose leptospirosis in the present study. In addition, patients with malaria or clinically obvious dengue virus infection were also excluded from the latter study [7]. Second, the current study was considerably influenced by an outbreak of dengue in 2012. The outbreak also contributed to a very much higher number of patients recruited in the second year compared to the first year of recruitment (Figure 3.1 and Figure 4.4). The data from national disease surveillance, Thailand, showed that there were peaks in the number of dengue cases in 2008, 2010, and 2013 [113]. Although, dengue was the major cause of AUFI in the present study, the proportion of cases was much lower than the study in 2008 [16]. Lastly, there was an outbreak of leptospirosis in Thailand between 1997 and 2002. The number of leptospirosis cases reported in Thailand was less than 200 per year prior to 1996 and was remarkably changed to over 2,000 cases in 1997 with a sudden increase up

to 14,285 cases in 2000. The extended outbreak occurred during 1997-2002 with a gradual decline to below 6,000 cases per year since [113].

Overall co-infections occurred in 1.7% of the non-malaria AUFI patients. Concomitant leptospirosis and scrub typhus infections accounted for almost all cases, occurring in 1.5% of patients. This co-infection has been reported previously in Thailand [7, 11, 275]. Co-infection may be common in areas that are exposed to multiple pathogens and share common risk factors. It is possible that the diagnostic tests play an important role in diagnosis of co-infection. Despite the development of molecular methods, serology assays remain the gold standard diagnostic test and have been widely used. The interpretation of serology assays is known to be problematic as antibodies from previous infections may persist and cross reactivity is common. Diagnosis of acute infection should be based on the rising antibody titres between paired acute and convalescent specimens [191]. Diagnostic accuracy of the assays depends on the timing of specimen collection and cut-off titres in which the optimal cut-off varies by prevalence of diseases in different geographical areas [207]. However, in the present study, molecular method (PCR) was used for diagnosis of acute leptospirosis infection from acute plasma specimen and IgM antibody detection was used for diagnosis of acute scrub typhus infection from paired acute and convalescent serum specimens. False positive scrub typhus results due to cross reactivity is a possibility, however, avoided for leptospirosis.

Despite the reference diagnostic tests used in the present study, the diagnoses for 65.4% of non-malaria AUFI patients remained unknown highlighting the importance of other pathogens in this population. Among these patients, 14.3% had only single acute specimen. The convalescent specimen could not be obtained because patients did not return for the follow-up visit or the patient died, although, the follow-up rate was very high in the current study (89.4%). The lack of convalescent specimens remains a major problem

in the serological diagnosis of infectious diseases, since the development of detectable antibody levels may take several days to weeks and antibodies are often undetectable in acute specimens, particularly when patients seek medical care early in the course of their illness. Molecular diagnostic tools such as PCR to detect nucleic acids of pathogens or rapid antigen detection on acute specimens are useful in this circumstance. Evaluations of such assays have been performed in this study and are described in the next chapters of this thesis.

The patients from the refugee clinic reported a higher proportion of confirmed diagnosis cases. Dengue and leptospirosis revealed seasonal variation in this study (Figure 4.4.). Dengue peaked between May and July, a similar time frame but narrower than the epidemiological trend in Thailand that showed a seasonal peak between May and September [276]. Leptospirosis peaked in the rainy season between July and October, which was also described elsewhere [115]. Cases of murine typhus and scrub typhus occurred throughout the study period with no distinguishing seasonal peak. Our results for scrub typhus contrasted with other reports where both scrub typhus and leptospirosis peaked in rice-growing seasons between July and November [7, 170]. On the other hand, murine typhus revealed a consistent result with a study of epidemiology of rickettsioses in Thailand, in which murine typhus was evenly diagnosed in all seasons [170]. The epidemiology and seasonality data of murine typhus in Thailand are scarce and are not well described. It is also not included in the Thai national reporting system [48].

Dengue, leptospirosis, murine typhus and scrub typhus share non-specific clinical presentations. Few clinical features and laboratory findings predict the cause of these infections. In the present study, younger age, red eyes, skin rash, cough (negative effect), lower neutrophil and lymphocyte counts and lower CRP concentration were independently associated with dengue. Leptospirosis was associated with younger age, location: refugee

clinic, shorter duration of fever and cough, lower platelet and higher CRP concentration. Murine typhus was associated with older age, longer duration of fever, and lower neutrophil counts, and scrub typhus was associated with longer duration of fever and higher CRP concentration. The results were different to a study of causes of non-malaria fever in Laos, which reported only platelet count (≤100,000/μl) was associated with dengue. An age of 15 years or younger, vomiting, cough, CRP level (>5 mg/l) were associated with leptospirosis. Female sex and hepatomegaly were associated with scrub typhus, and the association with murine typhus was not mentioned [12]. Studies in Thailand reported that dengue was associated with low platelet and WBC counts [16, 55]. The present study did not show that low platelet and WBC counts were associated with dengue to the same degree as the previous study in Thailand. That study described low platelet (<140,000/mm^3) and WBC counts (<5,000/mm^3) were strongly associated with dengue infection (odds ratio = 26.3 and 8.3, respectively) [55]. There are several explanations for this observation. Firstly, in the present study, patients presented early in the course of their fever when the reduction of platelets and WBC may not be observed [33]. Secondly, the previous study was focused on only dengue and scrub typhus so that could narrow down the variation factors in the analysis, whereas dengue cases in the present study were compared to non-dengue cases (including all other diagnoses and unknown diagnosis). In the current study, platelets were significantly lower in dengue compared to non-dengue cases in the univariate analysis but lost significance in the multivariate analysis. Eschar is the most important clinical sign that discriminates scrub typhus from other infectious diseases, but its presence is not consistent [170, 186, 188]. In the present study, an eschar was reported in only two patients. One patient had laboratory confirmed diagnosis of acute secondary dengue infection by paired serology (IgM/IgG ELISA, and PCR was also positive for DENV-1) and another patient had no diagnosis (negative for all available diagnostic tests). No patients with laboratory confirmed scrub

typhus had an eschar. This was likely to be due to clinicians not having the time or opportunity to examine patients thoroughly. Clinical diagnosis of murine typhus is more challenging since no specific clinical finding was reported. This emphasises the need for clinically useful rapid bedside diagnostic tests for these diseases.

The usefulness of non-specific infection markers (i.e. CBC and CRP) were assessed in the present study for differential diagnosis of dengue, leptospirosis, murine typhus and scrub typhus. Only the CRP concentration and WBC count were significantly different between these diseases. Although the WBC, neutrophil and lymphocyte counts showed significant differences between diagnosis groups, these differences were mostly within the normal ranges (WBC and neutrophil counts) and lower ranges (lymphocyte count). Therefore, they were not very useful for the differential diagnosis in this population.

The CRP result is useful to distinguish between acute viral and bacterial infections as confirmed in the present study (Figure 4.5). CRP is an established biomarker of bacterial infection and most of our patients presented early in the course of their fever (the median of two days), which is in the time of peak secretion of CRP (36-50 hours) [277]. Patients with diagnoses of dengue, leptospirosis, murine typhus and scrub typhus had CRP levels above 5.0 mg/l, which correlated to the expected normal values (<5.0 mg/l) according to the manufacturer's instructions. Results from the present study are in line with a recent large study that measured and compared CRP and procalcitonin levels to distinguish viral from bacterial and malarial causes of fever in patients from rural SE Asia. A subset of specimens from the present study was included in this publication and the results were comparable to specimens from Cambodia and Laos. This study revealed that CRP level was elevated in bacterial and malarial infections when compared with viral infections [268]. While the CRP level is able to discriminate viral from bacterial infection,

the cut-off value of CRP to differentiate between different diseases is uncertain and the optimal cut-off has not been established [268, 277].

In a malaria low transmission area like SE Asia, the proportion of sub-microscopic infections is high compared to the areas of high transmission intensity [238]. In the present study, sub-microscopic malaria infection was examined in non-malaria AUFI patients. Malaria smear and RDT were applied to rule out malaria patients at enrolment. Using sensitive qPCR to detect and quantify sub-microscopic infection, malaria DNA was detected in 17.5% of the patients and the parasite density was low with a geometric mean of 281.1 parasites/ml of blood. The sub-microscopic malaria infection might not be the cause of fever in the present study as the malaria parasitaemia was very low. A previous malaria epidemiological study from SMRU in a Karen population reported that the estimated pyrogenic parasite density was 1,460 parasites/µl for *P. falciparum* and 181 parasites/µl for *P. vivax*, although, the parasitaemia was determined by microscopy [237]. In addition, malaria DNA was detected in patients who already had confirmed diagnoses of the other causes of their illness (27.7% [44/159]). Although the majority of the sub-microscopic malaria infection was found in patients with unknown diagnosis, the overall parasite density was low in all diagnosis groups including the unknown diagnosis group. There were no differences in the parasite density between these groups (Figure 4.7) nor were there when comparing patients with known diagnosis and the no diagnosis group (Figure 4.8). There were three patients with unknown diagnosis who had high parasitaemia ($>10^6$ parasites/ml of blood), in which malaria could be the cause of their fever, however the number of patients with high parasitaemia was small and a definitive conclusion could not be made. There is no consensus threshold of parasite density for individuals to develop fever, although the symptomatic threshold for *P. falciparum* is known to be higher than for *P. vivax*. The threshold for *P. falciparum* has been established in malaria-naive host, which ranged between 1,900-10,700 parasites/µl, but this threshold is highly variable between

individuals. It is also different between different strains of *P. falciparum*. In endemic areas, individuals may not develop fever until the parasite density reaches 5,000-10,000 parasites/μl [278, 279]. Therefore, the significance of detecting sub-microscopic levels of malaria in the present study is unknown and warrants further investigation.

In the malaria part of the present study (PART II), co-infection with malaria was found in 8.3% (10/121) of the patients. Malaria and scrub typhus was the main co-infection accounting for 7.4% (9/121) and only one patient had malaria and dengue co-infection (0.8%). This is consistent with the previously published data on causes of fever in pregnancy on the Thailand-Myanmar border (SMRU clinics) where malaria and rickettsial co-infection was the most common, but with slightly more cases of murine typhus than scrub typhus (3 vs. 2 cases, respectively). Co-infection with dengue was also reported in one case [10]. Conversely, this differs from the data of another fever study in non-pregnant adults on the Thailand-Myanmar border where dual infections were common, and co-infections of malaria and leptospirosis were the most common [11]. These studies had different criteria for patient recruitment, duration of study, diagnostic tests and diagnosis algorithms that could impact on the detection of co-infections. The malaria part of the current study provided preliminary data for the rate of dual infections. A study that includes a larger number of malaria patients is required to confirm this finding. The proportions of patients with *P. vivax* and *P. falciparum* infections were equal (50% each), no mixed infection of different species was found by smear/RDT. Using the PCR method, the proportion of patients infected with *P. vivax* (45.5%) was higher than that of *P. falciparum* (35.5%), and mixed infections of *P. falciparum* and *P. vivax* was found in 16.5% (80% of the mixed infections were diagnosed with *P. falciparum* by microscope/RDT). This is in line with the previous reports in Thailand where *P. falciparum* was the predominant species in Thailand until 1998 when the proportion of *P. falciparum* and *P. vivax* became equal: *P. vivax* cases have been more frequent at some

points since then [14, 280, 281]. The mixed species infection of malaria is known to be common in endemic areas and the detection rate is higher when using PCR rather than microscopy. The most common mixed species infection is *P. falciparum* and *P. vivax*. The other mixed species such as mixed *P. falciparum* and *P. malariae*, and mixed *P. vivax* and *P. ovale* have been reported but rarely [280, 282, 283]. The levels of mixed *P. falciparum* and *P. vivax* infections recorded in Thailand were 0.3-0.7% of the patients with positive smears [281]. Higher levels of this mixed infection have been reported in the West border. In fact, malaria in Thailand has been limited to a few border areas, mainly Myanmar and Cambodia since 1950s [283]. The high level of mixed infection in the current study was confirmed by PCR and is consistent with that recorded from previously published data from SMRU [237, 283].

The PCR was not able to determine the species in three patients (two were *P. falciparum*, and one were *P. vivax* by microscopy or RDT). The first patient was positive only by RDT for *P. falciparum*. The 18S *rRNA* qPCR specific for *Plasmodium* sp. and the microsatellite nested-PCR for speciation were negative. Given the higher sensitivity of the qPCR compared to the RDT, this patient could have had a false positive RDT result due to persistence of HRP-II antigen that can remain detectable for weeks after acute infection. Alternatively, it could have been a false negative qPCR result resulting from extraction failure or presence of PCR inhibitors [239]. The second patient was positive for *P. falciparum* by microscopy and RDT, and the 18S *rRNA* qPCR was also positive with the parasitemia of 1.3 $x10^6$ parasites/ml of blood. The microsatellite nested-PCR was negative and there was insufficient specimen to repeat the test. The last patient had smear positive for *P. vivax* and the 18S *rRNA* qPCR was also positive with the parasitemia of 1.3 $x10^5$ parasites/ml of blood, however the microsatellite nested-PCR was unable to determine the species.

5 Clinical evaluation of diagnostic tests for early diagnosis of acute dengue infection from a single specimen

5.1 Introduction and aims

Dengue is an important and widespread mosquito-borne viral disease in humans, with a recent estimate of 390 million dengue infections globally each year, of which 96 million present as symptomatic infections [38]. It causes a wide range of clinical manifestations from mild to severe and life threatening. Clinical management of patients is based on supportive treatment since specific anti-viral treatment for dengue infection is not yet available [34]. Diagnosis of acute dengue infection using clinical symptoms and signs is difficult, since it is hard to distinguish from other common antibiotic treatable causes of acute febrile illness such as leptospirosis [284] and scrub typhus [55]. Confirmation of diagnosis usually relies on laboratory testing. Hence, a rapid and reliable laboratory diagnostic test is required during the acute phase of infection for early diagnosis of dengue virus infection to assist patient management.

Laboratory confirmation of dengue virus infection may involve a combination of multiple diagnostic tests to achieve accurate diagnosis. Diagnostic accuracy of the tests depends largely on timing of infection and timing of specimen collection. Currently, timely and clinically useful laboratory diagnosis of dengue virus infection relies on nucleic acid detection (rRT-PCR or RT-PCR) or NS1 antigen detection (ELISA or ICT) from a single specimen during the acute phase of infection [16, 33]. After the acute phase of infection, serology becomes more useful. Serological assays such as IgM and IgG antibody detection by ELISAs are widely used to confirm dengue infection and discriminate between primary and secondary infections [70]. These serological assays usually require paired acute and convalescent serum specimens to determine differences between IgM and IgG antibody

levels, causing a delay in diagnosis and therefore, is useful for confirmation of infection rather than early diagnosis that facilitates clinical management of patients.

In this chapter, the performance characteristics of two diagnostic tests (NS1 antigen and IgM antibody ICT, and one-step SYBR Green based rRT-PCR) were evaluated prospectively for the detection of dengue virus infection during the acute phase of infection from patients presenting with acute undifferentiated febrile illness in a rural SE Asian clinical setting. The aims were to:

1. Determine the diagnostic accuracy and clinical value of using a new generation rapid diagnostic test (IgM/IgG antibody plus NS1 antigen detection, a combined single ICT) to diagnose acute dengue virus infection.

2. Assess inter-operator variation for the interpretation of the ICT results.

3. Determine the diagnostic accuracy and clinical value of using a molecular diagnostic method (rRT-PCR) to diagnose acute dengue virus infection.

4. Evaluate the molecular diagnostic assay (rRT-PCR) as an alternative to the ICT, and as a possible replacement for the current serological methods as gold standard test for the confirmation of acute dengue virus infection.

5. Assess the impact of malaria infection (sub-microscopic) on the performance characteristics of the diagnostic tests under evaluation.

5.2 Materials and Methods

5.2.1 Patients and methods

The study was conducted between March 2011 and March 2013 in three SMRU outpatient clinics (OPD) on the Thailand-Myanmar border, as described previously in chapter 2. Briefly, any patients aged at least five years presenting to the clinics with a fever of at least 38°C of up to seven days' duration and with a negative result for malaria smear or RDT were recruited into the study. Patients who had a clear alternative clinical diagnosis such as pneumonia, urinary tract infection, chickenpox, measles or skin/soft tissue infection were excluded.

Blood specimens were collected for laboratory diagnosis as described in section 2.6. Acute plasma specimens were used for detection of dengue viral nucleic acid by one-step SYBR Green based rRT-PCR assay [16, 259] and for detection of dengue NS1 antigen and IgM antibody by ICT (SD BIOLINE Dengue Duo, Standard diagnostics, Inc.). This ICT contains NS1 antigen detection and IgM/IgG antibody detection in a single cassette. Only the results of NS1 antigen and IgM antibody detection were included in the current analysis as markers of the early diagnosis of acute dengue infection. The tests were read by three independent readers who did not confer and the results were blinded. Inter-operator variability in the reading of the test results between the three readers was analysed. The consensus results were then used for final interpretation. Paired acute and convalescent serum specimens were tested using the AFRIMS dengue virus and JE virus IgM and IgG antibody ELISAs as the reference tests [70, 258]. In addition to the diagnosis of acute dengue infection, the serotype of dengue virus (DENV-1 to 4) was identified using a nested-RT PCR assay [74, 80]. This assay was performed using the same RNA extracts of acute plasma specimens from all patients positive by the rRT-PCR assay.

The impact of malaria infection (sub-microscopic) on the performance characteristics of the dengue diagnostic tests under evaluation was assessed. The term "malaria infection" in this chapter refers to sub-microscopic malaria infection, in which the malarial DNA was detected using 18S *rRNA* qPCR assay [241]. The details of laboratory testing procedures and their interpretations were described in section 2.9.

The lower limit of detection (LoD) of the one-step SYBR Green based rRT-PCR assay was assessed to determine the laboratory sensitivity of the assay for the detection of dengue virus group. The dengue virus control strains were obtained from AFRIMS, consisting of DENV-1 (Hawaii strain), DENV-2 (New Guinea strain), DENV-3 (H87 strain), and DENV-4 (814669 strain). Ten-fold dilution series of DENV-1, DENV-2, and DENV-3, starting from 10,000 to 0.1 PFU/ml and 100,000 to 1.0 PFU/ml for DENV-4 were constructed and tested in duplicate. A DENV 1-4 mixture was used as positive control of the assay and its LoD was also determined using a 10-fold serial dilution of the DENV 1-4 mixture tested in duplicate. The dilution mixture was prepared starting from the mix of 1,000 PFU/ml of DENV-1 to 3 and 10,000 PFU/ml of DENV-4, then diluted 10-fold until the dilution mixture of 0.01 PFU/ml of DENV-1 to 3 and 0.1 PFU/ml of DENV-4. R^2 value (coefficient of correlation) obtained for the standard curve and efficiency of the PCR assay were reported. The laboratory specificity of the assay was not performed, because of the limitation of resources for the other positive control viruses. However, the original protocol stated that this primer set was highly specific and did not amplify West Nile virus (Eg 101 strain), yellow fever virus (17D vaccine strain) and JE virus (JaGAr strain) [259].

5.2.2 Data analysis

Statistical analyses were performed using STATA/SE 10.1 (StataCorp LP) and graphs were drawn using Microsoft Excel 2010 (Microsoft). Diagnostic accuracy of the tests under evaluation (NS1 antigen and IgM antibody ICT, and rRT-PCR on acute plasma specimen) was calculated by comparing the results of individual tests and combinations of multiple tests (combined using an "OR" operator) with the reference results (IgM and IgG antibody ELISAs on paired serum specimens). A 2 x 2 table was constructed, in which the reference results were cross-tabulated with the results of the tests under evaluation to determine the rate of true positive, true negative, false positive and false negative results. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) with 95% CI and area under ROC curve (AUC) were calculated using the "diagt" routine and roctab command in STATA [285]. The McNemar test was used to compare sensitivities of the tests. Kappa values were generated to determine the level of inter-operator variation in the reading of the NS1 antigen and IgM antibody ICT test results, and the agreement between clinical diagnosis and laboratory confirmed diagnosis of acute dengue virus infection [286]. To assess the impact of malaria infection on the performance characteristics of the dengue diagnostic tests, the sensitivity, specificity, PPV and NPV with 95% CI and the AUC were stratified for patients who were positive and negative for malaria qPCR separately using the "diagt" routine and roctab command in STATA [285].

5.3 Results

5.3.1 Patients

Patients who presented to the OPD clinics with acute undifferentiated febrile illness were investigated, and 908 patients were recruited into the study. Convalescent serum specimens were obtained from 89.4% (812/908) of the patients. Only patients with available paired acute and convalescent serum specimens were included in the further analysis.

Of the 812 patients included in the analysis, 61.0% (495/812) were male. The median age was 18 years (IQR 12-29, range 5-63) and the median temperature at presentation was 38.5°C (IQR 38.2-39.0). The median duration of fever at the time of first presentation was two days (IQR 2-3) and the median interval between obtaining initial acute-phase serum specimens and convalescent serum specimens was 14 days (IQR 14-14, range 9-29). A laboratory confirmed diagnosis of acute dengue virus infection was given to 17.7% (144/812) of patients using the results of the reference tests. Acute primary dengue infection was confirmed in 12 patients and 132 patients had acute secondary dengue infections (Table 5.1).

Table 5.1 Laboratory confirmed diagnosis of 812 febrile patients using reference tests.

Reference results[a]	Number of patients (%)
Acute primary dengue infection	12 (1.5)
Acute secondary dengue infection	132 (16.3)
Acute JE infection	6 (0.7)
Acute secondary flavivirus infection[b]	1 (0.1)
Recent dengue infection	1 (0.1)
Recent secondary dengue infection	2 (0.25)
No evidence of flavivirus infection	658 (81.0)
Total	**812 (100)**

[a]AFRIMS dengue virus and JE virus IgM and IgG antibody ELISAs on paired acute and convalescent serum specimens

[b]Other flaviviruses: anti-dengue IgM of <40 EIA units in acute specimen with an increase in anti-dengue or anti-JE IgG between acute and convalescent specimens to an absolute value of >100 units, but the anti-dengue IgM units were less than the anti-JE IgM unit in the convalescent specimen (Appendix 2).

Presumptive diagnoses were given at the clinics based on clinical presentations. Of the 812 patients, 24.0% (195/812) were clinically diagnosed with dengue virus infection. The agreement between clinical diagnosis and the laboratory confirmed diagnosis of acute dengue infection was poor (*Kappa*=0.4602) (Table 5.2). The clinical diagnosis for the patients with the laboratory confirmed diagnosis of acute dengue virus infection is shown in Table 5.3. Of the 144 laboratory confirmed acute dengue infections, 67.4% (97/144) of patients were clinically diagnosed with dengue at presentation. For the other 47 patients, 22 patients (15.3%) were diagnosed with unknown fever, 16 patients (11.1%) with

leptospirosis, five patients (3.5%) with typhoid, three patients (2.1%) with viral illness and one patient (0.7%) with scrub typhus.

Table 5.2 Clinical diagnosis and laboratory confirmed diagnosis of acute dengue virus infection.

Clinical diagnosis	Laboratory confirmed diagnosis		Total
	Dengue	Not dengue	
Dengue (%)	97 (67.4)	98 (14.7)	**195 (24.0)**
Not dengue (%)	47 (32.6)	570 (85.3)	**617 (76.0)**
Total (%)	**144 (100)**	**668 (100)**	**812 (100)**

Table 5.3 Clinical diagnosis for patients with a laboratory confirmed diagnosis of acute dengue virus infection.

Clinical diagnosis	Laboratory confirmed acute dengue cases (%)
Dengue	97 (67.4)
Unknown fever	22 (15.3)
Leptospirosis	16 (11.1)
Typhoid	5 (3.5)
Viral illness	3 (2.1)
Scrub typhus	1 (0.7)
Total	**144 (100)**

5.3.2 Lower limit of detection (LoD) of the one-step SYBR Green based rRT-PCR assay for universal detection of dengue viruses

The assay was able to detect all four serotypes of dengue virus, as it was designed for universal detection of dengue viruses. However, it was not designed for specific serotype detection and therefore was unable to distinguish between different serotypes. The laboratory sensitivity of the assay for the detection of dengue viruses using amplification curve and melting curve analysis are shown in Figure 5.1 and Figure 5.2. The assay validation was performed for 24 replicates i.e. each 10-fold serial dilution was tested in duplicate for 12 qPCR runs (3 runs for each of DENV-1 to -4). All replicates were positive for each dilution point (100% repeatability and reproducibility). The assay LoD was determined to be 0.03 PFU/reaction (or equivalent to 1 PFU/ml of plasma specimen) for the detection of DENV-1, DENV-2 and DENV-3; and 0.33 PFU/reaction (or equivalent to 10 PFU/ml) for DENV-4. The LoD of the DENV 1-4 mixture was similar to the LoD of the individual serotypes, in which it was between 0.03-0.33 PFU/reaction (or equivalent to 1-10 PFU/ml) (Figure 5.4). Following validation of the assay, an optimal fluorescence threshold was set at 14,000 for all PCR runs. Specific melting temperatures were between 80.0°C and 83.3°C.

Standard curves were generated from the amplification plots of each serotype representing DENV-1, DENV-2, DENV-3, DENV-4, and DENV 1-4 mixture. The average R^2 value of the standard curve for each serotype (DENV-1 to 4) and the DENV 1-4 mixture were 0.96, 0.97, 0.96, 0.94, and 0.93 respectively. The efficiency of the assay in detection of DENV-1, DENV-2, DENV-3, DENV-4 and DENV 1-4 mixture was 90.0, 91.7, 97.4, 106.8 and 127.3, respectively. Figure 5.3 and Figure 5.4 show examples of standard curves for a PCR run of each dengue serotype and the DENV 1-4 mixture.

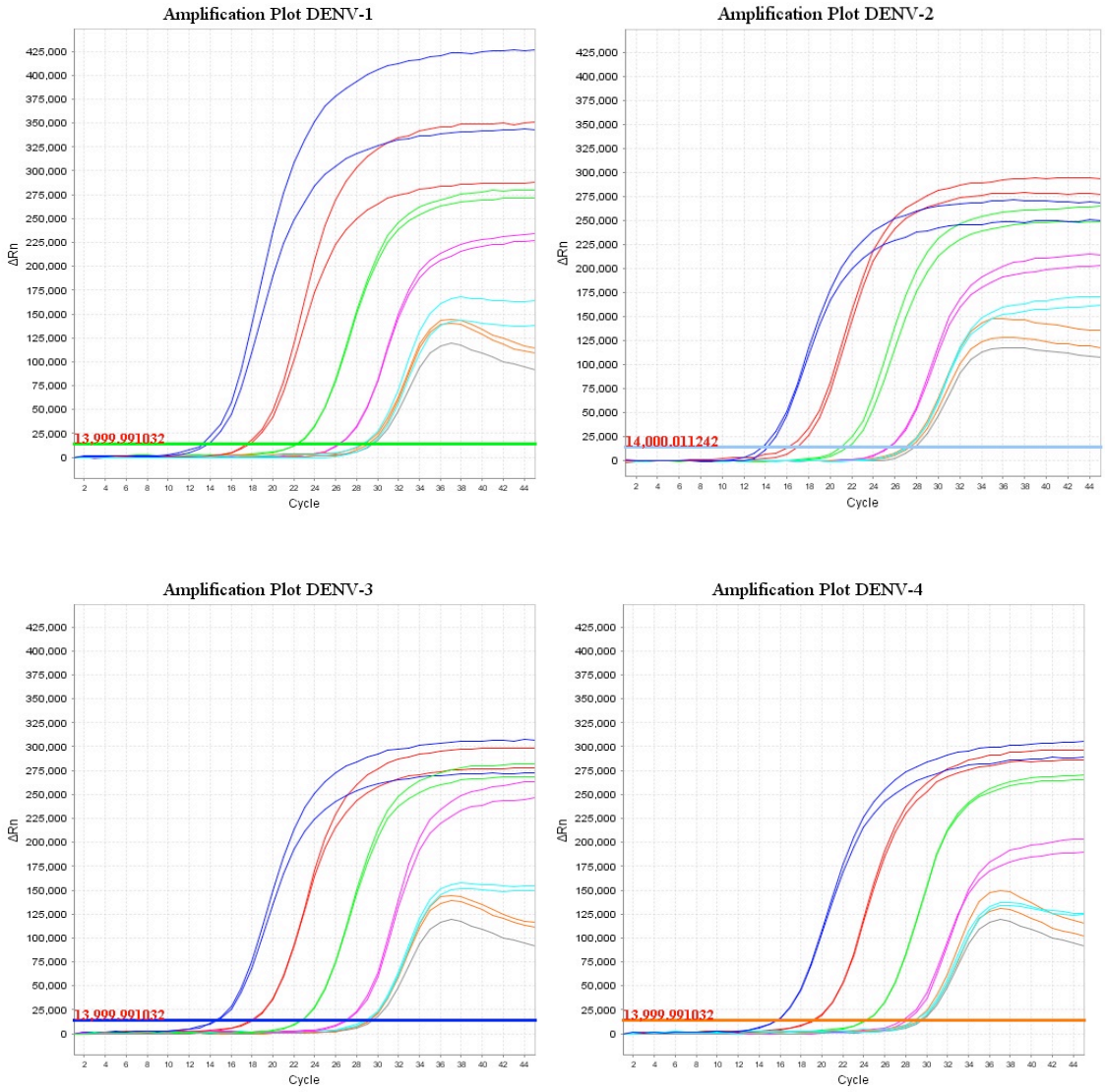


Figure 5.1 Amplification curves of the rRT-PCR assay for the detection of DENV-1, DENV-2, DENV-3, and DENV-4.

Legend: blue=333.33 PFU/reaction, red=33.33 PFU/reaction, green=3.33 PFU/reaction, pink=0.33 PFU/reaction, light blue=0.03 PFU/reaction, Orange=0.003 PFU/reaction, grey=negative control for DENV-1, DENV-2, and DENV-3

Legend: blue=3,333.33 PFU/reaction, red=333.33 PFU/reaction, green=33.33 PFU/reaction, pink=3.33 PFU/reaction, light blue=0.33 PFU/reaction, orange=0.03 PFU/reaction, grey=negative control for DENV-4

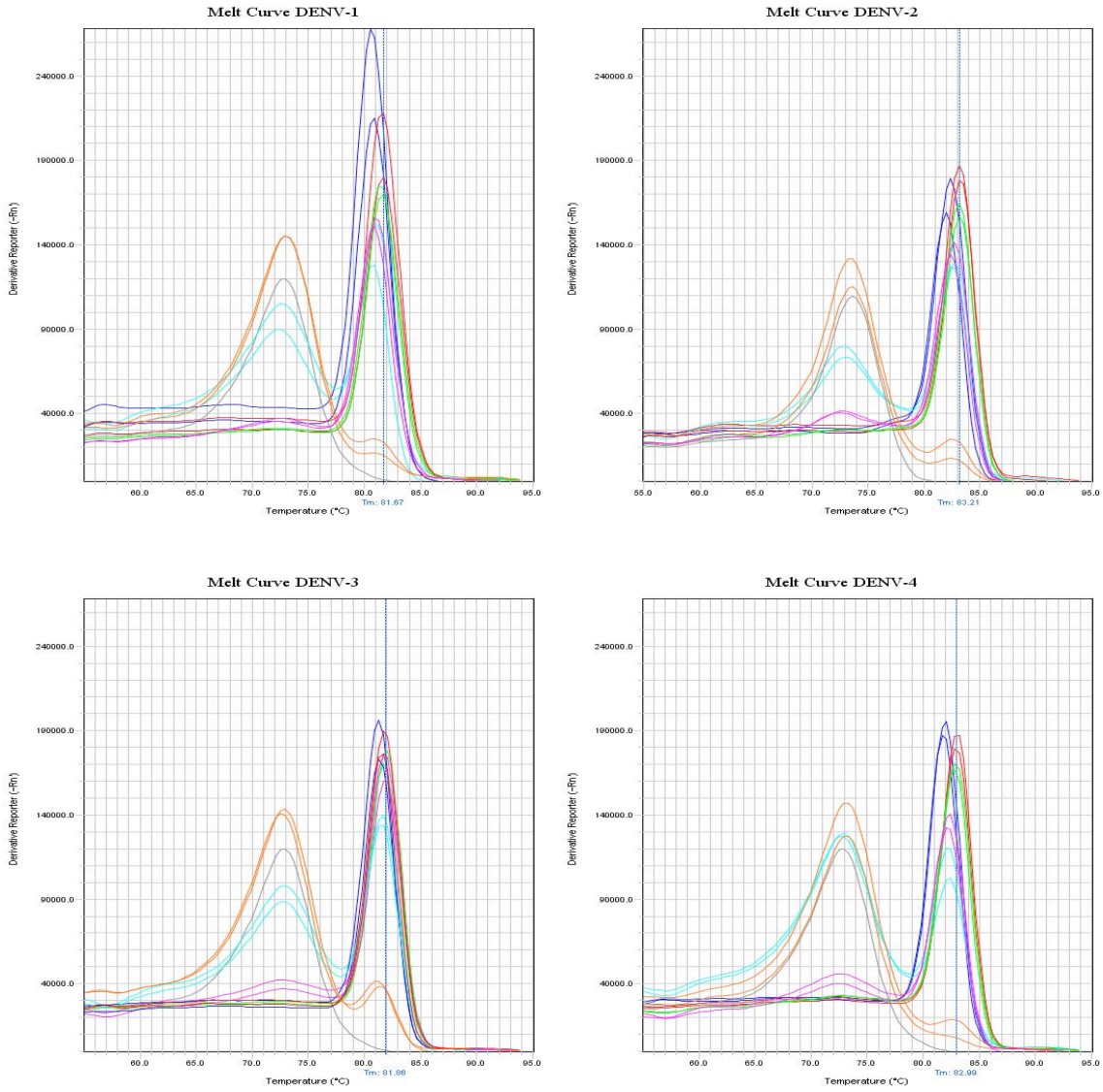


Figure 5.2 Melting curves of the rRT-PCR assay for the detection of DENV-1, DENV-2, DENV-3, and DENV-4.

Legend: blue=333.33 PFU/reaction, red=33.33 PFU/reaction, green=3.33 PFU/reaction, pink=0.33 PFU/reaction, light blue=0.03 PFU/reaction, Orange=0.003 PFU/reaction, grey=negative control for DENV-1, DENV-2, and DENV-3

Legend: blue=3,333.33 PFU/reaction, red=333.33 PFU/reaction, green=33.33 PFU/reaction, pink=3.33 PFU/reaction, light blue=0.33 PFU/reaction, orange=0.03 PFU/reaction, grey=negative control for DENV-4

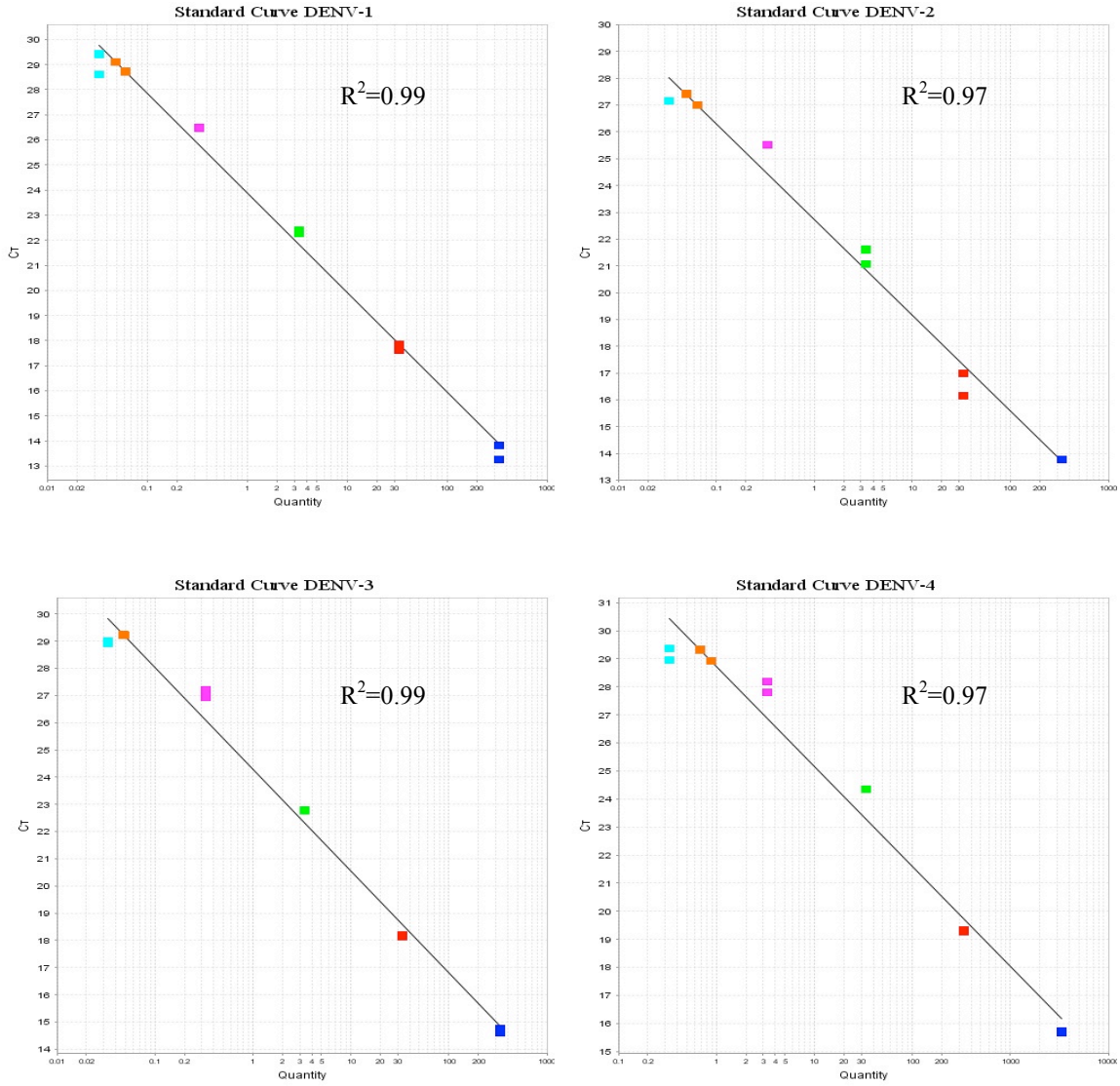


Figure 5.3 Standard curves of the rRT-PCR assay for the detection of DENV-1, DENV-2, DENV-3, and DENV-4.

Legend: blue=333.33 PFU/reaction, red=33.33 PFU/reaction, green=3.33 PFU/reaction, pink=0.33 PFU/reaction, light blue=0.03 PFU/reaction, Orange=0.003 PFU/reaction, grey=negative control for DENV-1, DENV-2, and DENV-3

Legend: blue=3,333.33 PFU/reaction, red=333.33 PFU/reaction, green=33.33 PFU/reaction, pink=3.33 PFU/reaction, light blue=0.33 PFU/reaction, orange=0.03 PFU/reaction, grey=negative control for DENV-4

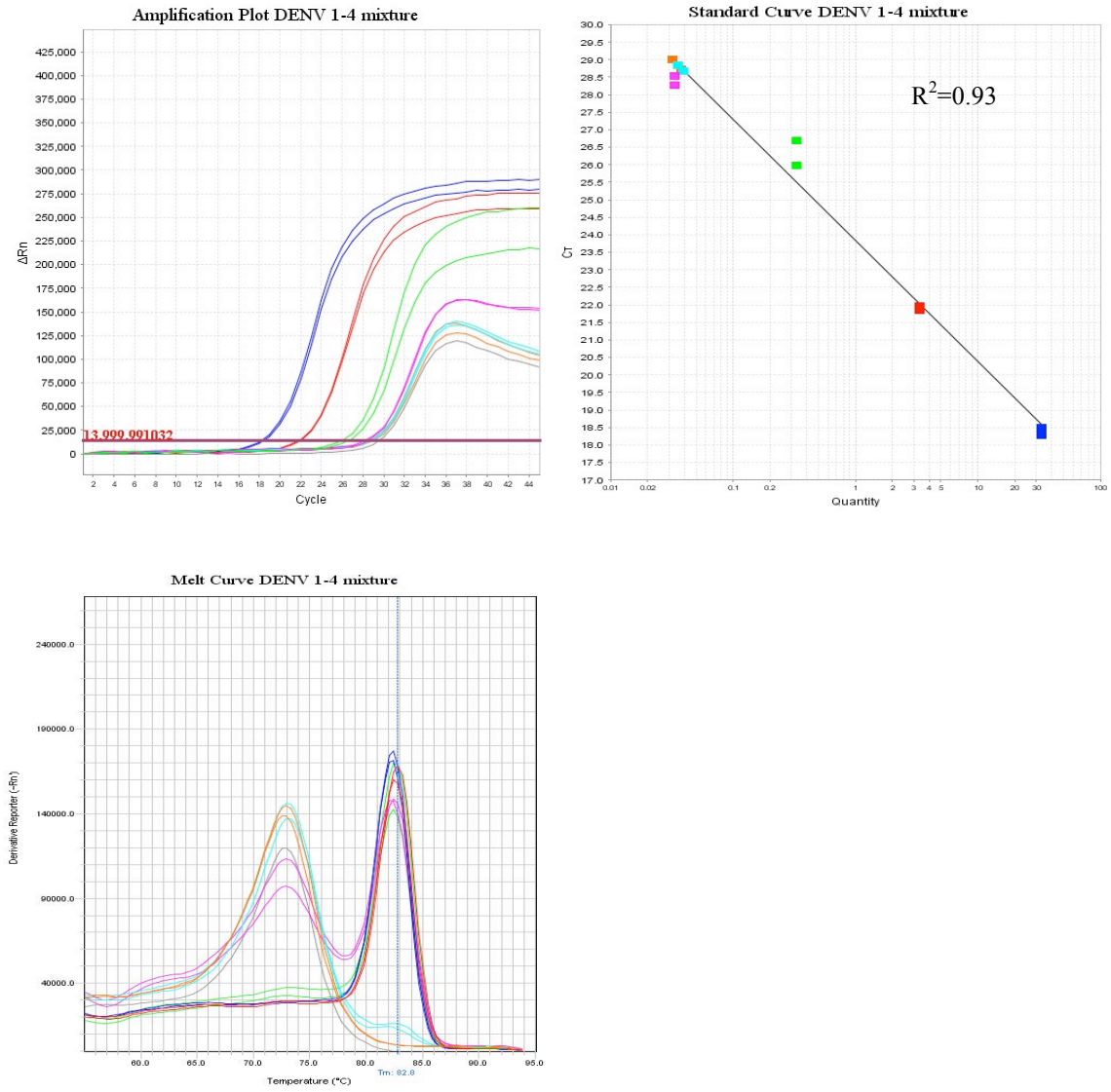


Figure 5.4 Amplification curve, standard curve and melting curve analysis of the rRT-PCR assay for the detection of DENV 1-4 mixture.

Legend: blue=33.33-333.33 PFU/reaction, red=3.33-33.33 PFU/reaction, green=0.33-3.33 PFU/reaction, pink=0.03-0.33 PFU/reaction, light blue=0.003-0.03 PFU/reaction, orange=0.0003-0.003 PFU/reaction, grey=negative control (the former number is for DENV-1 to 3 and the latter is for DENV-4)

5.3.3 Detection of dengue virus by rRT-PCR assay

Acute plasma specimens from 812 patients were tested using the rRT-PCR assay for the detection of dengue virus RNA. Of these, 18.0% (146/812) of the patients were given a positive result in which a specific melting temperature between 80.0-83.3°C was obtained. The median Ct value for these positive specimens was 19.7 (IQR 17.7-22.5, range 12.7-31.7). There was always some non-specific amplification in negative specimens, however no specific melting temperatures were obtained and the Ct values of the negative specimens (666/812) were late with the median of 30.0 (IQR 29.6-30.4, range 28.2-34.2).

In addition, the serotype of dengue virus (DENV-1 to 4) was identified by nested-RT PCR assay using the RNA extracts from the 146 patients that were positive by the rRT-PCR assay. All specimens from 146 patients were also positive by the nested-RT PCR and the serotypes were identified. Of these, 134 patients (91.8%) had DENV-1, eight patients (5.5%) had DENV-3 and four patients (2.7%) had DENV-4. No patients with DENV-2 infection were identified during the study period.

5.3.4 Detection of dengue virus by the Dengue Duo ICT

The SD BIOLINE Dengue Duo ICT (Standard diagnostics, Inc.) was performed on all 812 acute plasma specimens. The overall results of the tests, both NS1 antigen and IgM antibody detections, read by the three independent readers were similar as shown in Table 5.4.

Table 5.4 NS1 antigen and IgM antibody Dengue Duo ICT results (n=812) read by three independent readers.

Tests	Reader 1		Reader 2		Reader 3		Final results	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
NS1	130	682	130	682	130	682	130	682
IgM	40	772	40	772	40	772	40	772

5.3.5 Inter-operator variation for interpretation of the Dengue Duo ICT results

The inter-operator variation for interpretation of the ICT results was assessed using Kappa statistics. The results of both NS1 antigen detection and IgM antibody detection were easy to read and reliable for different readers. There was excellent inter-operator agreement amongst three readers for both assays (*Kappa*=1.00, and 0.96 for NS1 antigen detection and IgM antibody detection, respectively). The consensus results (i.e. 2/3 readers with the same interpretation) were then used for the final results (Table 5.4) and further analysis.

5.3.6 Diagnostic accuracy of the individual tests

The performance characteristics of rRT-PCR, NS1 antigen detection and IgM antibody detection on the acute plasma specimen compared to the reference tests are shown in Table 5.5. rRT-PCR or NS1 antigen detection alone had high sensitivity, 97.2% and 86.1% respectively. The sensitivity of rRT-PCR was significantly higher than that of the NS1 antigen detection (P=0.0003). The sensitivity of IgM antibody detection alone was low (7.6%), which was significantly lower than both rRT-PCR (P<0.0001) and NS1 antigen detection (P<0.0001).

Specificities of rRT-PCR, NS1 antigen detection and IgM antibody detection were 99.1%, 99.1%, and 95.7% respectively. The diagnostic accuracy of the tests was also

measured by the area under ROC curve (AUC). The AUC of rRT-PCR and NS1 antigen detection were excellent (0.98 and 0.93, respectively), but poor for IgM antibody detection (0.52) (Table 5.5).

5.3.7 Diagnostic accuracy of the combinations of the tests

The performance characteristics of the combinations of the tests on acute plasma specimens are shown in Table 5.5. These included the combination of NS1 antigen detection and IgM antibody detection from the ICT (NS1+IgM), and the combination of rRT-PCR and the ICT (rRT-PCR+NS1+IgM). The overall sensitivity of the combination of rRT-PCR+NS1+IgM was 98.6%, which was significantly higher than the sensitivity of the NS1+IgM combination (86.1%) or NS1 antigen detection or IgM antibody detection alone (all $P<0.0001$), but not significantly higher than the rRT-PCR alone ($P=0.5000$). The combination of NS1+IgM was significantly more sensitive than IgM antibody detection alone ($P<0.0001$), but not significantly different from the NS1 antigen detection alone ($P=1.0000$). The sensitivity of the NS1+IgM combination was significantly lower than that of rRT-PCR alone ($P=0.0004$).

The overall specificity of the combinations of the tests was slightly lower compared to the individual tests. The specificity of the combinations of rRT-PCR+NS1+IgM, and NS1+IgM tests was 94.5%, and 94.9%, respectively. The AUC for both combinations was excellent with 0.97 and 0.91 for the combination of rRT-PCR+NS1+IgM, and NS1+IgM, respectively (Table 5.5).

5.3.8 Effect of fever duration at presentation on diagnostic test sensitivity for early diagnosis of acute dengue infection

The effect of fever duration at the time of presentation on test sensitivity is shown in Figure 5.5. The sensitivity of rRT-PCR alone (83.3-100%) and the combination of rRT-PCR+NS1+IgM was high throughout the early stage of infection (92.9-100%). The

sensitivity of NS1 antigen detection was also high throughout, but was lower than rRT-PCR for the detection in patients presenting with one to four days of fever (78.6-87.8%). The sensitivity of IgM antibody detection alone was low and increased later, peaking in patients presenting with five days of fever (33.3%). There was no positive IgM antibody detection in patients presenting with six days of fever. The sensitivity of NS1 antigen detection combined with IgM antibody detection was covered by the NS1 antigen detection, in which the sensitivity was similar to the NS1 antigen detection alone. The numbers of patients presenting with six and seven days of fever were small, and therefore the 95% confidence intervals around the sensitivities were wide.

Table 5.5 Performance characteristics of rRT-PCR, NS1 antigen detection, and IgM antibody detection on acute plasma specimens compared to reference tests for early diagnosis of acute dengue infection (n=812).

Tests		Reference results*		%Sensitivity (95% CI)	%Specificity (95% CI)	%PPV (95% CI)	%NPV (95% CI)	AUC
		Dengue	Not dengue					
rRT-PCR	+	140	6	97.2 (93.0-99.2)	99.1 (98.1-99.7)	95.9 (91.3-98.5)	99.4 (98.5-99.8)	0.9816
	-	4	662					
NS1	+	124	6	86.1 (79.4-91.3)	99.1 (98.1-99.7)	95.4 (90.2-98.3)	97.1 (95.5-98.2)	0.9261
	-	20	662					
IgM	+	11	29	7.6 (3.9-13.3)	95.7 (93.8-97.1)	27.5 (14.6-43.9)	82.8 (79.9-85.4)	0.5165
	-	133	639					
rRT-PCR+NS1+IgM	+	142	37	98.6 (95.1-99.8)	94.5 (92.4-96.1)	79.3 (72.7-85.0)	99.7 (98.9-100)	0.9654
	-	2	631					
NS1+IgM	+	124	34	86.1 (79.4-91.3)	94.9 (93.0-96.4)	78.5 (71.2-84.6)	96.9 (95.3-98.1)	0.9051
	-	20	634					

AUC= area under ROC curve, CI= confidence interval, NPV= negative predictive value, PPV= positive predictive value, rRT-PCR= real-time reverse transcriptase PCR

*AFRIMS dengue virus and JE virus IgM and IgG antibody ELISAs on paired acute and convalescent serum specimens

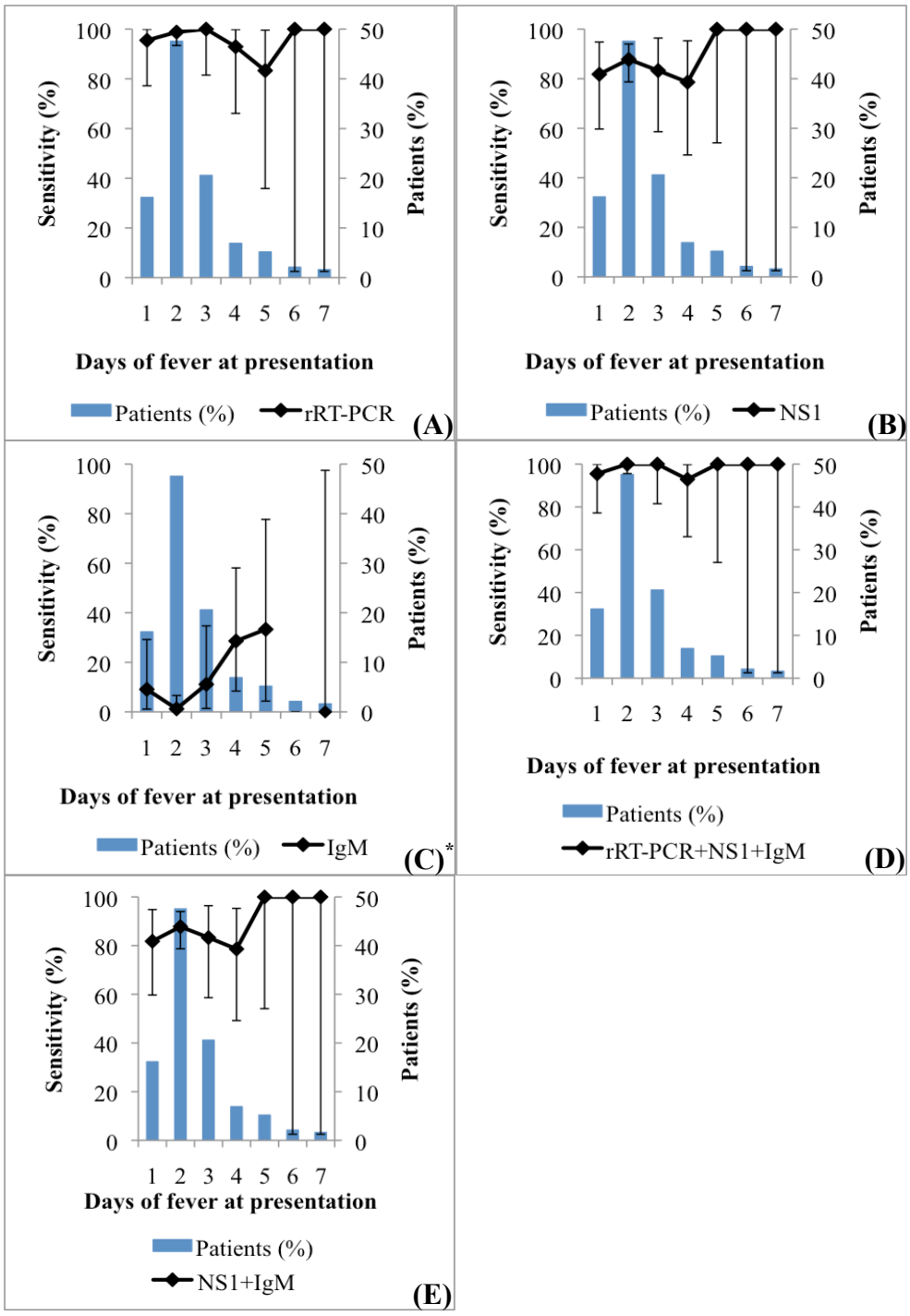


Figure 5.5 Effect of fever duration at presentation on sensitivity of the tests for early diagnosis of acute dengue infection (n=812).

(A) rRT-PCR, (B) NS1 antigen, (C) IgM antibody, (D) combination of rRT-PCR, NS1 antigen and IgM antibody, (E) combination of NS1 antigen and IgM antibody

*No positive IgM antibody detection in patients presenting with six days of fever

5.3.9 Impact of malaria infection on the diagnostic accuracy of the tests for early diagnosis of acute dengue infection

From a total of 812 non-malaria febrile patients in the current analysis, malarial DNA was detected in 139 patients by 18S *rRNA* qPCR assay (sub-microscopic malaria infection). Malarial DNA was detected in 12.5% (18/144) of patients with laboratory confirmed diagnosis of acute dengue infection and was detected in 18.1% (121/668) of patients in whom the reference tests did not confirm acute dengue infection. The impact of malaria infection on the performance characteristics of the tests under evaluation for early diagnosis of acute dengue infection was assessed by stratified diagnostic accuracies of the tests in malaria qPCR positive patients (Table 5.6) and malaria qPCR negative patients (Table 5.7). The overall diagnostic accuracy of all individual and combination tests was similar between malaria qPCR positive and negative patients. The sensitivity of all tests was slightly higher in malaria qPCR negative patients compared to malaria qPCR positive patients, however the differences were not statistically significant. Similarly, the specificity of all tests was slightly better in patients with malaria qPCR positive, although they were not statistically different. There was insufficient evidence to determine if malaria infection had a significant impact on the accuracy of the diagnostic tests evaluated in this study. Therefore, the malaria infection had no impact or might have only a minimal impact that was not clinically important on the performance characteristics of the dengue diagnostic tests.

Table 5.6 Impact of malaria infection on the performance characteristics of the tests for early diagnosis of acute dengue infection in malaria qPCR positive patients (n=139).

Tests		Reference results*		%Sensitivity (95% CI)	%Specificity (95% CI)	%PPV (95% CI)	%NPV (95% CI)	AUC
		Dengue	Not dengue					
rRT-PCR	+	17	0	94.4 (72.7-99.9)	100 (97.0-100)	100 (80.5-100)	99.2 (95.5-100)	0.9722
	-	1	121					
NS1	+	15	1	83.3 (58.6-96.4)	99.2 (95.5-100)	93.8 (69.8-99.8)	97.6 (93.0-99.5)	0.9125
	-	3	120					
IgM	+	0	8	0 (0-18.5)	93.4 (87.4-97.1)	0 (0-36.9)	86.3 (79.2-91.6)	0.4669
	-	18	113					
rRT-PCR+NS1+IgM	+	17	8	94.4 (72.7-99.9)	93.4 (87.4-97.1)	68.0 (46.5-85.1)	99.1 (95.2-100)	0.9392
	-	1	113					
NS1+IgM	+	15	8	83.3 (58.6-96.4)	93.4 (87.4-97.1)	65.2 (42.7-83.6)	97.4 (92.6-99.5)	0.8836
	-	3	113					

AUC= area under ROC curve, CI= confidence interval, NPV= negative predictive value, PPV= positive predictive value, rRT-PCR= real-time reverse transcriptase PCR

*AFRIMS dengue virus and JE virus IgM and IgG antibody ELISAs on paired acute and convalescent serum specimens

Table 5.7 Impact of malaria infection on the performance characteristics of the tests for early diagnosis of acute dengue infection in malaria qPCR negative patients (n=673).

Tests		Reference results*		%Sensitivity (95% CI)	%Specificity (95% CI)	%PPV (95% CI)	%NPV (95% CI)	AUC
		Dengue	Not dengue					
rRT-PCR	+	123	6	97.6 (93.2-99.5)	98.9 (97.6-99.6)	95.3 (90.2-98.3)	99.4 (98.4-99.9)	0.9826
	-	3	541					
NS1	+	109	5	86.5 (79.3-91.9)	99.1 (97.9-99.7)	95.6 (90.1-98.6)	97.0 (95.2-98.2)	0.9280
	-	17	542					
IgM	+	11	21	8.7 (4.4-15.1)	96.2 (94.2-97.6)	34.4 (18.6-53.2)	82.1 (78.9-85.0)	0.5245
	-	115	526					
rRT-PCR+NS1+IgM	+	125	29	99.2 (95.7-100)	94.7 (92.5-96.4)	81.2 (74.1-87.0)	99.8 (98.9-100)	0.9695
	-	1	518					
NS1+IgM	+	109	26	86.5 (79.3-91.9)	95.2 (93.1-96.9)	80.7 (73.1-87.0)	96.8 (95.0-98.1)	0.9088
	-	17	521					

AUC= area under ROC curve, CI= confidence interval, NPV= negative predictive value, PPV= positive predictive value, rRT-PCR= real-time reverse transcriptase PCR

*AFRIMS dengue virus and JE virus IgM and IgG antibody ELISAs on paired acute and convalescent serum specimens

5.4 Discussion

Accurate early diagnosis of acute dengue virus infection remains challenging in SE Asia where dengue is endemic. Clinical presentations are often confused with other causes of acute undifferentiated fever and diagnosis based on clinical features is non-specific [16, 33], as shown in the current study where only 49.7% (97/195) of patients who were clinically diagnosed with dengue had laboratory confirmed diagnosis of acute dengue virus infection.

In the current study, the diagnostic accuracy and clinical usefulness of the rRT-PCR assay, and NS1 antigen and IgM antibody detection ICT were evaluated for the timely diagnosis of acute dengue infection from a single plasma specimen. The overall performance characteristics of the IgM antibody detection were poor with a sensitivity of 7.6%, although the specificity was high (95.7%). The positive predictive value (PPV) was 27.5% and the negative predictive (NPV) value was 82.8%, resulting in many misdiagnosed cases if the test were to be used in absence of the NS1 positive line in the ICT cassette. There are several explanations for this observation. Firstly, most of the patients presented early in the course of their illness with a median of two days (IQR 2-3) of fever, when the IgM antibody level was undetectable. Secondly, the majority of the dengue confirmed cases were secondary infections in which the IgM antibody levels are variable and might be undetectable [24]. The NS1 antigen and IgM antibody detection ICT had excellent inter-operator agreement between readers. This means that the results are easy to interpret and reliable for different readers. The feasibility assessment of using this ICT in the field has been described elsewhere. This ICT appeared to be stable in the tropical field where the ambient temperature is high. [287].

Following the validation of the rRT-PCR assay, the analytical sensitivity was between 0.03 and 0.33 PFU/reaction (or equivalent to 1-10 PFU/ml of plasma specimen)

which was better than that stated in the original protocol (between 4.1 and 43.5 PFU/ml) [259]. Both the rRT-PCR and NS1 antigen detection had excellent diagnostic accuracy (Table 5.5) and either could potentially be used as a single test for early confirmation of acute dengue infection. The rRT-PCR was the most accurate test (97.2% sensitivity and 99.1% specificity) and had slightly better performance than the NS1 antigen detection (86.1% sensitivity and 99.1% specificity, and the AUC was 0.98 vs. 0.92, respectively). In practice, the NS1 antigen detection and the IgM/IgG antibody detection were on the same ICT cassette, although different strips (one for NS1 antigen and another one for IgM/IgG antibody detection). The ICT used ~120µl of acute plasma specimen and the results were available in 15 minutes. The cost of this ICT was 450 THB per test (13.2 USD). The rRT-PCR used 200µl of the acute plasma specimen and the result was available in around 7 hours from receipt in the laboratory (1.5 hours for nucleic acid extraction, 4.5 hours for rRT-PCR and one hour for setting up, interpretation and reporting). The cost of rRT-PCR plus nucleic acid extraction was 700 THB per specimen (20.5 USD). For the combinations of the tests, the combination of rRT-PCR+NS1+IgM had very high sensitivity, although the specificity decreased compared to individual tests. Combining the IgM antibody with the NS1 antigen detection, the sensitivity was not better than the NS1 antigen detection alone and resulted in an inevitable drop in the specificity.

The clinical diagnostic accuracy of the rRT-PCR assay and NS1 antigen detection rapid test have been evaluated and reported in many studies for the acute diagnosis of dengue from a single specimen [16, 71, 259, 288, 289]. In the current study, the sensitivity of rRT-PCR (97.2%) was slightly better than the original protocol, which found that the assay could only detect dengue in 83.0% of acute serum specimens from confirmed dengue cases [259]. This is also true for the previous fever diagnostic study at SMRU where the sensitivity of the rRT-PCR alone was 88.9% for the acute diagnosis of dengue infection from acute plasma specimens [16]. Interestingly, given that most of the patients had acute

secondary dengue infection in the current study, NS1 antigen detection ICT had high sensitivity (86.1%). Teoh and colleagues reported the sensitivity of NS1 antigen detection (NS1 Ag Strip) was 95.9% for the primary dengue infection and 43.7% for the secondary dengue infection. This may be explained because patients in the current study presented to the clinics very early in the course of their fever with a median of two days illness (IQR 2-3, range 1-7) while the median duration of fever from the study of Teoh *et al* was six days (range 1 to ≥11) [288].

The impact of malaria infection on the diagnostic accuracy of the tests was assessed. Importantly, rRT-PCR and NS1 antigen detection were highly sensitive and specific independent of malaria infection status. This is also true for the combination of NS1+IgM ICT and rRT-PCR+NS1+IgM, which were covered by the NS1 antigen detection or rRT-PCR. The IgM antibody detection alone had high specificity but low sensitivity also independent of the malaria infection status. Indeed, the cross reactivity between dengue and malaria on the dengue IgM antibody detection rapid tests has been described elsewhere [73, 290], however, malaria infection status did not have an impact on the IgM antibody detection in this evaluation. This could be due to the low malaria parasite density of sub-microscopic infection as described in chapter 4.

The current study has several limitations. Analysis of the performance characteristics of the diagnostic tests comparing primary (n=12) and secondary (n=132) dengue infection was not performed, since the number of patients with confirmed diagnosis of acute primary dengue infection was small. However, this reflects the situation in most resource-limited clinical settings as well as the situation in most dengue endemic areas like SE Asia, specifically Thailand where almost 90% of dengue cases are secondary infection [41]. The evaluation of the diagnostic tests comparing all four serotypes of dengue virus could not be performed because all serotypes were not represented in the study. Over 90% of cases were

DENV-1 and none were DENV-2. The performance of diagnostic tests depends on the timing of infection and timing of specimen collection. Since patients in this study presented to the clinics very early in the course of their illness, the analysis was limited to the small number of patients who presented after five days duration of fever. This may affect the performance characteristics of the tests, particularly IgM antibody detection, in which its detectable levels appear during this time and peak slightly later for primary infection and the lower levels for secondary infection [24]. The nucleic acid detection (RT-PCR) assay could detect dengue viral RNA from first day of fever until seven days [259]. NS1 antigen could be detected from blood on the first day after onset of fever up to day nine of fever [61].

The present study has demonstrated that the rRT-PCR or new generation rapid ICT test that combines NS1 antigen and IgM/IgG antibody detection can reliably be used as a single test in a clinical setting for the early diagnosis of acute dengue infection from a single specimen. The rRT-PCR was the most accurate test in this evaluation, however it was more expensive and took longer to get the result than using a rapid test. In addition, it requires a considerable investment for setting up a laboratory, especially where the system is not already in place. This study has confirmed the findings of the previous pilot study at SMRU as well as provided more useful information with a larger sample size and a better reflection of the general situation of AUFI in a rural SE Asian clinical setting. This study was specifically looking at the performance characteristics of a clinic bedside diagnostic test rather than a laboratory based diagnostic test as in the previous study [16]. In the previous study, evaluations of NS1 antigen detection and IgM antibody detection were performed in an ELISA format. Although it is standardised and inexpensive, the turnaround time is longer than the ICT format and this new generation ICT was unavailable at the time. The overall performance of NS1 antigen detection ICT in this evaluation (86.1% sensitivity and 99.1% specificity) was better than the ELISA used in the

previous study (54.2% sensitivity and 100% specificity). For the IgM antibody detection, the ICT had lower sensitivity (7.6%), but higher specificity (95.7%) compared to the previously evaluated ELISA (16.7% sensitivity and 87.8% specificity). However, the sensitivity was poor for both evaluations. Indeed, the rRT-PCR and NS1 antigen detection have proven useful with high diagnostic accuracy and ability to be used as a single test for early diagnosis. The design of the previous study did not provide information on the impact of malaria infection on the diagnostic accuracy of the test. Therefore, the data from the current study suggests that in a clinical setting in rural areas where resources are limited, this new generation ICT is useful to facilitate the management of patients. The rRT-PCR could replace the gold standard serology for early diagnosis during the acute phase of dengue infection.

6 Clinical evaluation of diagnostic tests for early diagnosis of acute scrub typhus and murine typhus infections from a single specimen

6.1 Introduction and aims

Scrub typhus and murine typhus are important acute febrile illnesses in the Asia Pacific region and are endemic in Thailand [7, 10]. Clinical diagnosis of rickettsial infections is difficult because early symptoms are similar to other regionally endemic infections such as dengue, leptospirosis, and malaria [11]. Scrub typhus, caused by *Orientia tsutsugamushi*, and murine typhus, caused by *Rickettsia typhi*, are obligate intracellular Gram-negative bacteria which are transmitted to humans by the bite of trombiculid mites (chigger) and rat fleas (mainly *Xenopsylla cheopis*), respectively [167]. The organisms are difficult to isolate in-vitro from clinical specimens, requiring prolonged culture and biosafety level-3 (BSL-3) precautions [194]. Thus, isolation is not appropriate for routine clinical laboratory diagnosis. Serological testing, using the IFA for the detection of rising antibody titres on paired acute and convalescent specimens, is the current serological gold standard for confirmation of rickettsial infection [196]. However, serology is not useful for early diagnosis since antibodies are rarely detectable during the acute phase of illness, a convalescent specimen is required for accurate diagnosis especially in an endemic setting and there is no consensus on how to interpret the results based on a single acute specimen [191]. Many rapid bedside diagnostic tests based on the detection of antibodies were developed and evaluated for the clinical usefulness of early diagnosis for scrub typhus and murine typhus infection [208, 209, 291, 292], but their availability or diagnostic accuracy is inadequate. Given the limitations of serology, many molecular based methods have been developed for rapid detection of rickettsial species [210, 220] from different blood fractions, as well as skin or tissue [212, 213, 216, 219], to

enable detection of bacterial DNA prior to the development of antibodies and give a result in time to assist patient management during the acute phase of illness.

In this chapter, the performance characteristics of three diagnostic tests: SD BIOLINE Scrub typhus IgM ICT (Standard diagnostics, Inc.) and 47kDa qPCR assay for scrub typhus, and 17kDa qPCR assay for murine typhus were evaluated prospectively. The tests were evaluated using a single specimen during the acute phase of infection from patients presenting with AUFI in a rural SE Asian clinical setting. The aims were to:

1. Determine the diagnostic accuracy and clinical value of using the SD BIOLINE Scrub typhus IgM antibody detection ICT (Standard diagnostics, Inc.) to diagnose acute scrub typhus infection.

2. Assess inter-operator variation for the interpretation of the ICT results.

3. Determine the diagnostic accuracy and clinical value of using molecular diagnostic assays to diagnose acute scrub typhus (47kDa qPCR assay) and acute murine typhus (17kDa qPCR assay) infections.

4. Evaluate the 47kDa qPCR assay as an alternative to the ICT, and as a possible replacement for the current serological methods as gold standard tests for the confirmation of acute scrub typhus infection.

5. Evaluate the 17kDa qPCR assay as a possible method to replace the current serological methods as gold standard tests for the confirmation of acute *Rickettsia* spp. (mainly murine typhus) infection.

6. Assess the impact of malaria infection (sub-microscopic) on the performance characteristics of the diagnostic tests under evaluation.

6.2 Materials and Methods

6.2.1 Patient specimens and methods

All available paired acute and convalescent serum specimens from non-malaria febrile patients were included in this evaluation. Blood specimens were collected for laboratory confirmed diagnosis and diagnostic test evaluation as described in section 2.6. Acute plasma specimens were tested using SD BIOLINE Scrub typhus IgM ICT (Standard diagnostics, Inc.) for detection of IgM antibody against *O. tsutsugamushi.* The tests were read by three independent readers who did not confer and the results were blinded. Inter-operator variability in the reading of the test results between three readers was analysed. After that, the consensus results were used for the final interpretation of the test results. Acute buffy coat specimens were tested using 47kDa and 17kDa qPCR assays for detection of *O. tsutsugamushi* and *Rickettsia* spp. DNA, respectively [210, 217]. All positive specimens for 17kDa qPCR assay were tested further by *ompB* qPCR assay for detection of *R. typhi* DNA [218]. Specimens positive for 17kDa and *ompB* qPCR assays were defined as positive for murine typhus infection. Any specimens positive for 17kDa qPCR assay but negative for *ompB* qPCR assay were sent for sequencing (Macrogen, Seoul, Republic of Korea) for confirmation of rickettsial species. Paired serum specimens were tested using the in-house ELISA/IFA assays (MORU) for detection of IgM antibody against *O. tsutsugamushi* and *R. typhi* as the reference tests [207, 208, 261-263].

The LoD of the 47kDa, 17kDa, and *ompB* qPCR assays was assessed to determine the laboratory sensitivity of the assays for the detection of *O. tsutsugamushi*, *Rickettsia* spp., and *R. typhi*, respectively. Plasmid controls were obtained from *O. tsutsugamushi* strain UT76 (47kDa gene), and *R. typhi* strain Wilmington (17kDa and *ompB* genes). For each assay, a 10-fold serial dilution of linearised plasmid DNA positive control starting from 100,000 to 1.0 copies/µl was constructed and tested in duplicate. R^2 value (coefficient

of correlation) obtained for the standard curve and efficiency of the qPCR assays were reported. The laboratory specificity of the three assays was determined by testing with 1.0 ng/µl of the following organisms: *Enterococcus faecalis*, *E. coli*, *P. falciparum*, *P. vivax*, *Pseudomonas aeruginosa*, *S. aureus*, *Streptococcus* Group B and *S. pneumoniae* and 3.3-33.3 PFU/µl of DENV1-4 mix.

Bacterial loads were calculated with the following formula: number of the target DNA copies/ml of blood = [(number of copies/µl of DNA template)/2] x 100. Numbers of copies/µl of DNA template were calculated using 10-fold serial dilution of linearised plasmid DNA (100,000-10 copies/µl), resulting in numbers of copies/µl of DNA extract. The factor 2 adjusted for the 1:2 ratio of DNA extract to buffy coat, resulting in numbers of copies/µl of buffy coat. The factor 100 corrected for the buffy coat fraction, which made up approximately 10% of the whole blood [293].

In addition, the impact of malaria infection on the performance characteristics of the scrub typhus and murine typhus diagnostic tests for early diagnosis of acute phase of infection was assessed. In this chapter, the term "malaria infection" refers to sub-microscopic malaria infection, in which the malarial DNA was detected using 18S *rRNA* qPCR assay [241]. The details of laboratory testing and their interpretations were described in section 2.9.

6.2.2 Data analysis

Data were analysed using STATA/SE 10.1 (StataCorp LP) and graphs were created using Microsoft Excel 2010 (Microsoft). Diagnostic accuracy of the tests under evaluation (SD BIOLINE Scrub typhus IgM ICT (Standard diagnostics, Inc.), 47kDa qPCR, and 17kDa PCR assays) was calculated by comparing the results of individual tests and a combination of the tests (combined using an "OR" operator) with the reference results (IgM antibody ELISA/IFA on paired serum specimens). The reference results were cross-

tabulated with the results of the diagnostic tests under evaluation to determine the rate of true positive, true negative, false positive and false negative results. The sensitivity, specificity, PPV, NPV with 95% CI and AUC were calculated using the "diagt" routine and roctab command in the STATA [285]. The McNemar test was used to compare sensitivity of the tests. Kappa values were generated to determine the level of inter-operator variation in the reading of the SD BIOLOINE Scrub typhus IgM ICT results (Standard diagnostics, Inc.) and the agreement between clinical diagnosis and laboratory confirmed diagnosis of acute scrub typhus and murine typhus infections [286]. The impact of malaria infection on the performance characteristics of the scrub typhus and murine typhus diagnostic tests were assessed by stratifying the sensitivity, specificity, PPV, NPV with 95% CI and AUC for patients with malaria qPCR positive and negative groups using the "diagt" routine and roctab command in STATA [285].

6.3 Results

6.3.1 Patient specimens

Paired acute and convalescent serum specimens were available from 89.3% (811/908) of the non-malaria febrile patients and were included in the analysis. A laboratory confirmed diagnosis of acute scrub typhus was given to 5.4% (44/811) of patients and acute murine typhus was confirmed in 6.7% (54/811) of the patients by the results of the reference tests.

Presumptive diagnoses were given at the clinics based on the clinical presentations. Of the 811 patients, 1.6% (13/811) were clinically diagnosed with scrub typhus infection. The agreement between clinical diagnosis and the laboratory confirmed diagnosis of acute scrub typhus infection was poor (*Kappa* = 0.0106) (Table 6.1). None of the patients were given a clinical diagnosis of murine typhus.

The clinical diagnoses for patients with the laboratory confirmed diagnosis of acute scrub typhus and acute murine typhus infections are shown in Table 6.2. Of the 44 patients with laboratory confirmed acute scrub typhus infection, only one patient was clinically diagnosed with scrub typhus at presentation.

Table 6.1 Clinical diagnosis and laboratory confirmed diagnosis of acute scrub typhus infection.

Clinical diagnosis	**Laboratory confirmed diagnosis**		**Total**
	Scrub typhus	**Not scrub typhus**	
Scrub typhus (%)	1 (2.3)	12 (1.6)	**13 (1.6)**
Not scrub typhus (%)	43 (97.7)	755 (98.4)	**798 (98.4)**
Total (%)	**44 (100)**	**767 (100)**	**811 (100)**

Table 6.2 Clinical diagnoses for patients with the laboratory confirmed diagnosis of acute scrub typhus and acute murine typhus infections.

Clinical diagnosis	**Laboratory confirmed diagnosis**	
	Acute scrub typhus (%)	**Acute murine typhus (%)**
Dengue	5 (11.4)	4 (7.4)
Leptospirosis	9 (20.5)	17 (31.5)
Scrub typhus	1 (2.3)	1 (1.9)
Typhoid	9 (20.5)	5 (9.3)
Unknown fever	20 (45.5)	27 (50.0)
Total	**44 (100)**	**54 (100)**

6.3.2 Laboratory sensitivity and specificity of 47kDa, 17kDa and *ompB* qPCR assays

The laboratory sensitivity of the 47kDa, 17kDa and *ompB* qPCR assays for the detection of the *O. tsutsugamushi*, *Rickettsia spp.*, and *R. typhi*, respectively, was determined using 10-fold dilution series of linearised plasmid DNA. Amplification curves were plotted as shown in Figure 6.1. The accurate quantification could be calculated using the data points between 10-100,000 copies/µl (five points). The assay validation was performed for eight replicates for 47kDa assay (each 10-fold serial dilution was tested in duplicate for four qPCR runs), eight replicates for 17kDa assay (each 10-fold serial dilution was tested in duplicate for four qPCR runs) and six replicates for *ompB* assay (each 10-fold serial dilution was tested in duplicate for three qPCR runs). All replicates were positive for each dilution point for the three assays (100% repeatability and reproducibility). The LoD of the three assays was similar, which was determined to be between 1-10 copies/µl of DNA template (or equivalent to 50-500 copies/ml of blood). Following validation of the assays, an optimal fluorescence threshold was set at 25,000 for 47kDa qPCR assay, and 30,000 for 17kDa and *ompB* qPCR assays for all qPCR runs. A standard curve was generated from the amplification plots of each assay. The average R^2 values of the standard curves were 1.00 for all three assays. The efficiency of the 47kDa, 17kDa and *ompB* assays was 89.4, 87.8 and 98.8%, respectively. Figure 6.2 shows examples of standard curves for a qPCR run of each assay. For the laboratory specificity, the three qPCR assays were specific. There were no amplifications for all other organisms tested.

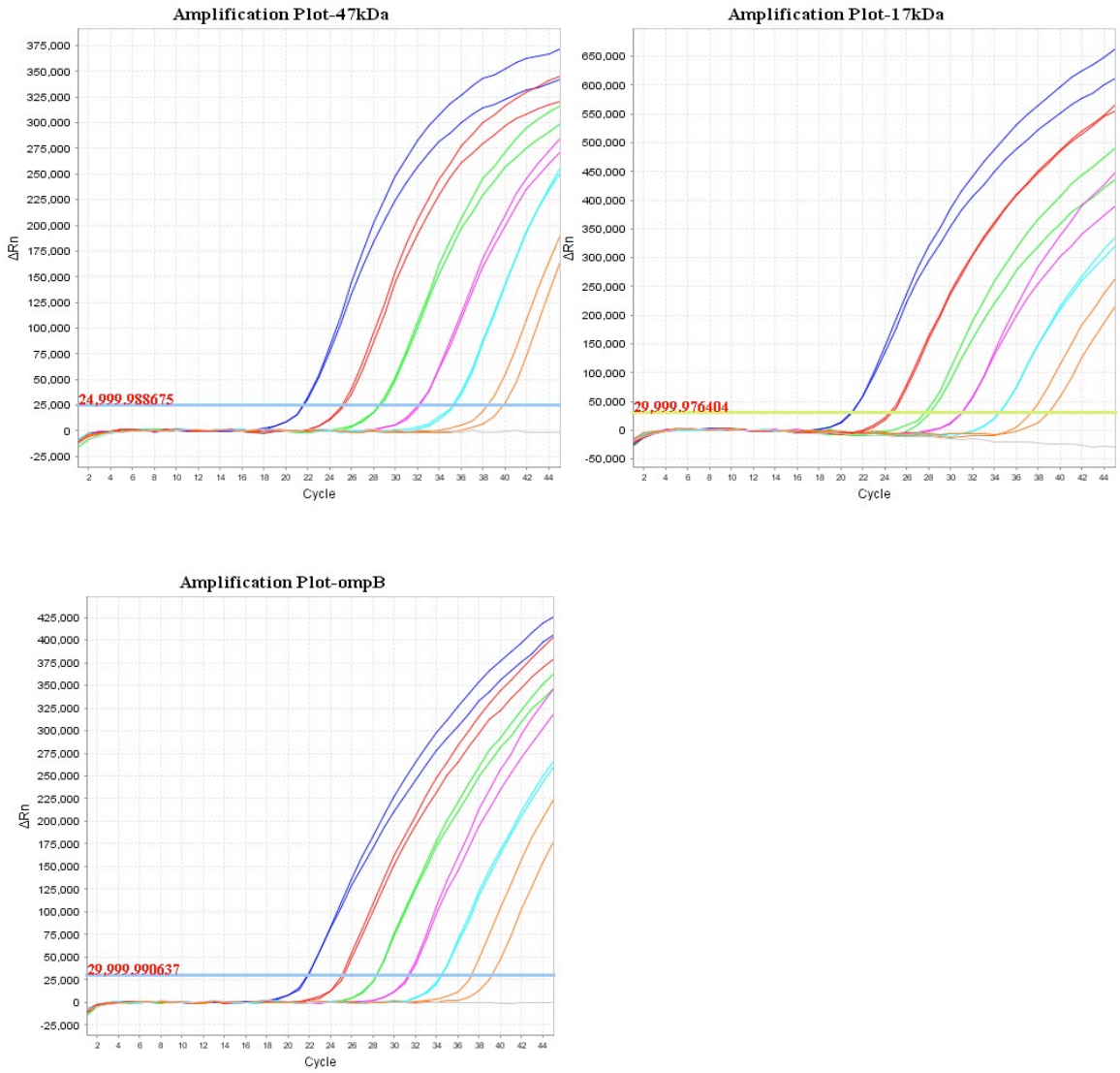


Figure 6.1 Amplification curves of the 47kDa, 17kDa and *ompB* qPCR assays for the detection of *O. tsutsugamushi*, *Rickettsia* spp., and *R. typhi*, respectively, using 10-fold serial dilution of linearised plasmid DNA.

Legend: blue=100,000 copies/µl, red=10,000 copies/µl, green=1,000 copies/µl, pink=100 copies/µl, light blue= 10 copies/µl, orange=1 copy/µl, and grey=negative control

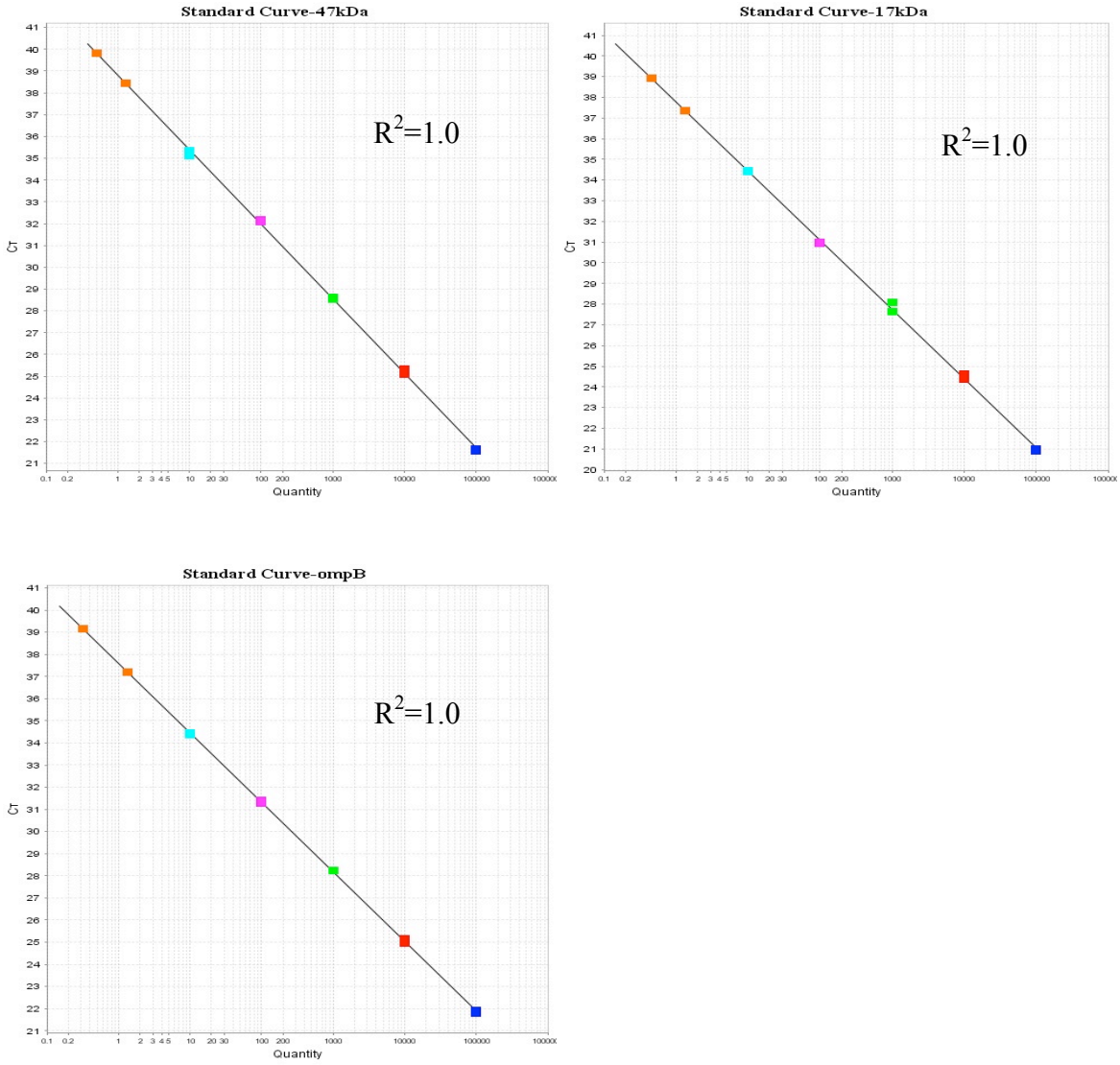


Figure 6.2 Standard curves of the 47kDa, 17kDa and *ompB* qPCR assays for the detection of *O. tsutsugamushi*, *Rickettsia* spp., and *R. typhi,* respectively, using 10-fold serial dilution of linearised plasmid DNA.

Legend: blue=100,000 copies/µl, red=10,000 copies/µl, green=1,000 copies/µl, pink=100 copies/µl, light blue=10 copies/µl, orange=1 copy/µl, and grey=negative control

6.3.3 Detection of *O. tsutsugamushi* DNA by 47kDa qPCR assay

Acute buffy coat specimens from 811 patients were tested using the 47kDa qPCR assay for the detection of *O tsutsugamushi* DNA. Of these, 1.6% (13/811) of the patients were given a positive result.

Quantification of the *O. tsutsugamushi* DNA in positive acute buffy coat specimens was obtained from 4/13 specimens using the 47kDa qPCR assay. The copy number ranged from 12.0 to 25.6 copies/µl of DNA extract (600 to 1,280 copies/ml of blood) with a median of 18.1 copies/µl of DNA extract (905 copies/ml of blood). The quantification of the other 9/13 positive specimens was lower than the accurate quantification threshold (<10 copies/µl of DNA extract). Thus, the actual quantification was not reported (Table 6.3). Of all 13 patients with acute buffy coat specimens positive in the 47kDa qPCR assay, 10 patients were confirmed as having acute scrub typhus infection by the results of the reference tests, and the other three positives were from non-acute scrub typhus cases (no diagnosis). Of 10 confirmed scrub typhus cases, the quantification of the *O. tsutsugamushi* DNA was obtained from 3/10 specimens. The copy number ranged from 12.0 to 22.4 copies/µl of DNA extract (600 to 1,120 copies/ml of blood) with a median of 13.8 copies/µl of DNA extract (690 copies/ml of blood). The quantification of the other 7/10 positive specimens was not reported, since they were lower than the accurate quantification threshold (<10 copies/µl of DNA extract). For the three positive specimens from non-acute scrub typhus cases, the quantification was obtained from only one specimen which was 25.6 copies/µl of DNA extract (1,280 copies/ml of blood).

Table 6.3 Quantification of *O. tsutsugamushi* DNA in positive acute buffy coat specimens using 47kDa qPCR assay.

	Number of 47kDa qPCR positive (%)	Median copies/µl of DNA extract (range)
All positive 47kDa qPCR	4 (30.8)	18.1 (12.0-25.6)
	9 (69.2)	<10
	13 (100)	
Acute scrub typhus*	3 (30.0)	13.8 (12.0-22.4)
	7 (70.0)	<10
	10 (100)	
Not acute scrub typhus	1 (33.3)	25.6 (25.6-25.6)
	2 (66.7)	<10
	3 (100)	

47kDa qPCR= quantitative real-time PCR targeting 47kDa

*Confirmed diagnosis of acute scrub typhus infection by IgM ELISA/IFA using paired acute and convalescent specimens

6.3.4 Detection of scrub typhus IgM antibody by the Scrub typhus IgM ICT

The SD BIOLINE Scrub typhus IgM ICT (Standard diagnostics, Inc.) was available for 810 tests and these results were included in the current analysis. The overall results of the test read by three independent readers were slightly different as shown in Table 6.4.

Table 6.4 Scrub typhus IgM antibody ICT results (n=810) read by three independent readers.

Tests	Reader 1		Reader 2		Reader 3		Final results	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
IgM	62	748	63	747	71	739	62	748

6.3.5 Inter-operator variation for interpretation of the Scrub typhus IgM ICT results

The inter-operator variation in reading the results of the ICT was assessed using Kappa statistics. The result of the test was easy to read. There was excellent inter-operator agreement amongst three readers (*Kappa*=0.9445). The consensus results (i.e. 2/3 readers with the same interpretation) were then used for the final results (Table 6.4) and further analysis.

6.3.6 Diagnostic accuracy of scrub typhus diagnostic tests

The performance characteristics of 47kDa qPCR assay and scrub typhus IgM antibody detection ICT (IgM ICT) for early diagnosis of the scrub typhus infection on the acute specimens compared to the reference tests are shown in Table 6.5. Both tests had high specificity (99.6 and 93.5% for 47kDa qPCR and IgM ICT, respectively), but poor sensitivity (22.7 and 27.3% for 47kDa qPCR and IgM ICT, respectively). The sensitivity improved when combining the 47kDa qPCR and the IgM ICT (36.4%), although, the specificity fell slightly compared to the 47kDa qPCR alone, but it remained high (93.2%). There was no difference between the sensitivity of the 47kDa qPCR and the IgM ICT (*P*=0.5217). The sensitivity of the combination of 47kDa qPCR and IgM ICT (47kDa qPCR+IgM ICT) was significantly higher than the 47kDa qPCR or the IgM ICT alone

(*P*=0.0143 and *P*=0.0455). The AUC of 47kDa qPCR, IgM ICT, and combination of 47kDa qPCR+IgM ICT were poor (0.61, 0.60, and 0.65, respectively).

Correlation between the IgM ICT results and IgM IFA titres on acute specimens from patients with confirmed acute scrub typhus infection is shown in Table 6.6. Most patients with confirmed acute scrub typhus infection had low IgM IFA titres of ≤1:400 in the acute specimens (42/44 patients) and this correlated with the positivity rate of the IgM ICT, which was low in these specimens. The positivity rate of the IgM ICT was higher in acute specimens with high IgM IFA titres, however the number of patients with high IgM IFA titres on acute specimens was small.

Table 6.5 Performance characteristics of the scrub typhus IgM ICT and 47kDa qPCR on acute specimens compared to reference tests for early diagnosis of acute scrub typhus infection (n=810).

Tests		Reference results*		%Sensitivity (95% CI)	%Specificity (95% CI)	%PPV (95% CI)	%NPV (95% CI)	AUC
		Scrub typhus	Not scrub typhus					
47kDa qPCR	+	10	3	22.7 (11.5-37.8)	99.6 (98.9-99.9)	76.9 (46.2-95.0)	95.7 (94.1-97.0)	0.6117
	-	34	763					
IgM ICT	+	12	50	27.3 (15.0-42.8)	93.5 (91.5-95.1)	19.4 (10.4-31.4)	95.7 (94.0-97.1)	0.6037
	-	32	716					
47kDa qPCR+IgM ICT	+	16	52	36.4 (22.4-52.2)	93.2 (91.2-94.9)	23.5 (14.1-35.4)	96.2 (94.6-97.5)	0.6479
	-	28	714					

47kDa qPCR= quantitative real-time PCR targeting 47kDa, AUC= area under ROC curve, CI= confidence interval, IgM ICT= IgM antibody detection immunochromatographic test, NPV= negative predictive value, PPV= positive predictive value

*IgM ELISA/IFA using paired acute and convalescent specimens

Table 6.6 Correlation between scrub typhus IgM antibody detection ICT results and IgM IFA titres on acute specimens from patients with confirmed acute scrub typhus infection* (n=44).

IgM IFA titres	Scrub typhus IgM ICT results		Total
	Negative (%)	Positive (%)	
<1:100	17 (89.5)	2 (10.5)	19
1:100	7 (70.0)	3 (30.0)	10
1:200	7 (87.5)	1 (12.5)	8
1:400	1 (20.0)	4 (80.0)	5
1:800	0 (0)	1 (100)	1
1:25600	0 (0)	1 (100)	1
Total	**32 (72.7)**	**12 (27.3)**	**44**

IgM ICT= IgM antibody detection immunochromatographic test, IgM IFA= IgM antibody detection indirect immunofluorescent assay

*Confirmed diagnosis of acute scrub typhus infection by IgM ELISA/IFA using paired acute and convalescent specimens

6.3.7 Detection of *Rickettsia* spp. and *R. typhi* by 17kDa and *ompB* qPCR assays/sequencing confirmed *R. typhi*

Acute buffy coat specimens from 811 patients were tested using the 17kDa qPCR assay for the detection of *Rickettsia* spp. DNA. Of these, 2.8% (23/811) of the patients were given a positive result.

All DNA extracts from 23 acute buffy coat specimens that were positive by the 17kDa qPCR assay were then tested using *ompB* qPCR assay for the detection of *R. typhi* DNA. Of these, 78.3% (18/23) of the patients were given a positive result. For the other

five *ompB* negative specimens, one was identified as *R. typhi*, one was *R. felis*, and three were unable to be identified by sequencing, since good quality DNA could not be obtained (very low positive).

Therefore, there were a total of 19 patients with acute buffy coat specimens positive for 17kDa qPCR assay and confirmed to have *R. typhi* by *ompB* qPCR/sequencing assay. Of these, 17 patients were confirmed to have acute murine typhus infection by the reference tests and two patients were not confirmed to have acute murine typhus infection. All 17kDa qPCR positive specimens were determined to have less than 10 copies/µl of DNA extract, below the accurate quantification threshold.

6.3.8 Diagnostic accuracy of murine typhus diagnostic test

For murine typhus, the performance characteristics of the 17kDa qPCR assay were evaluated. This assay was performed as a screening test for the detection of *Ricksttsia* spp. and the *ompB* qPCR assay was performed on all specimens that were positive by the 17kDa qPCR assay to identify the detection of *R. typhi*. Therefore, only specimens that were positive for both 17kDa qPCR and *ompB* qPCR/sequencing confirmed *R. typhi* were included in the current analysis. The performance characteristics of the 17kDa qPCR assay for the early diagnosis of the acute murine typhus infection on the acute specimens compared to the reference tests are shown in Table 6.7. The 17kDa qPCR had excellent specificity (99.7%), but the sensitivity was poor (31.5%). The AUC of the assay was also poor (0.66).

Table 6.7 Performance characteristics of the 17kDa qPCR on acute specimens compared to reference tests for early diagnosis of acute murine typhus infection (n=811).

Test		Reference results[b]		%Sensitivity (95% CI)	%Specificity (95% CI)	%PPV (95% CI)	%NPV (95% CI)	AUC
		Murine typhus	Not Murine typhus					
17kDa qPCR[a]	+	17	2	31.5 (19.5-45.6)	99.7 (99.0-100)	89.5 (66.9-98.7)	95.3 (93.6-96.7)	0.6561
	-	37	755					

17kDa qPCR= quantitative real-time PCR targeting 17kDa, AUC= area under ROC curve, CI= confidence interval, NPV= negative predictive value, PPV= positive predictive value

[a]Specimens positive for 17kDa and *ompB* qPCR assays/sequencing confirmed *R. typhi*

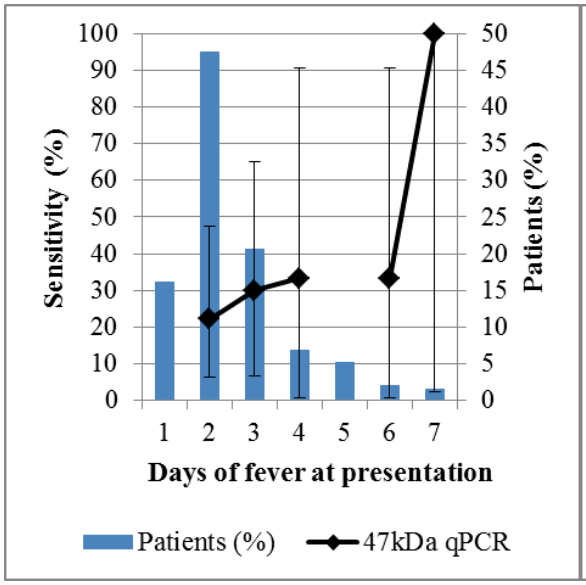
[b]IgM ELISA/IFA using paired acute and convalescent specimens

6.3.9 Effect of fever duration at presentation on diagnostic test sensitivity for early diagnosis of acute scrub typhus and acute murine typhus infections

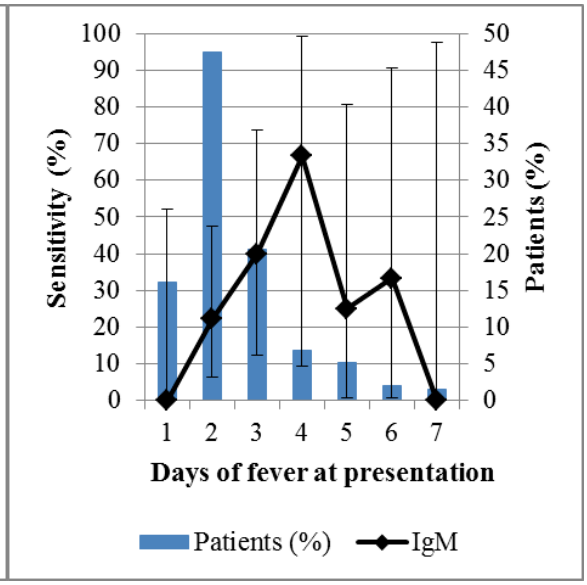
For scrub typhus infection, the effect of fever duration at the time of presentation on the sensitivity of the diagnostic tests is shown in Figure 6.3. For the 47kDa qPCR assay alone, there were no positive specimens in patients presenting with one and five days of fever. The sensitivity of the 47kDa qPCR in patients presenting between two and four days and at six days of fever was around 20-30%. The sensitivity peaked in patients presenting with seven days of fever, although the numbers of patients presenting with six and seven days of fever were small. For the IgM ICT alone, the sensitivity was low at the beginning and increased later, peaking in patients presenting with four days of fever (66.7%).

The sensitivity of the 47kDa qPCR+IgM ICT combination was improved in patients presenting with two days of fever (38.9%) and covered the gap for patients presenting with one and five days of fever when the 47kDa qPCR alone was unable to detect scrub typhus infection. The overall numbers of patients presenting after three days of fever were small and the confidence intervals around the sensitivity were very wide (Figure 6.3).

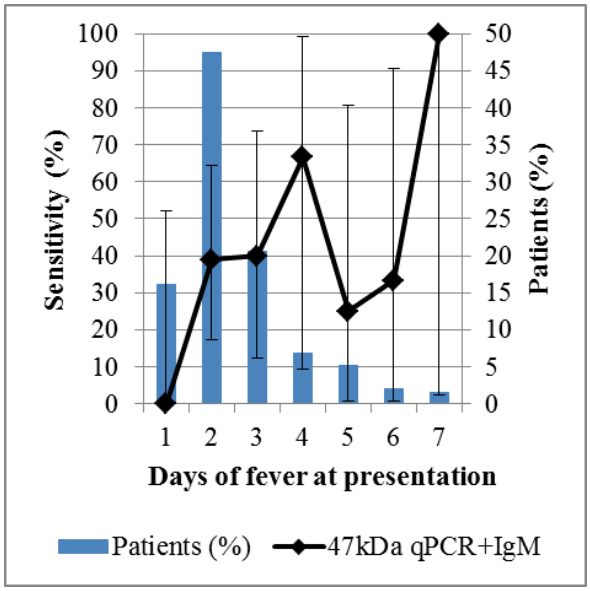
For murine typhus infection, the sensitivity of the 17kDa qPCR assay varied between 25.0 and 55.6% by duration of fever. The sensitivity was 50.0% in patients presenting with one day of fever. The sensitivity decreased to below 30.0% in patients presenting with two and three days of fever, and increased again in patients with four and five days of fever (55.6%). The sensitivity slightly decreased again in patients presenting with six days of fever (33.3%). There were no positive specimens for 17kDa qPCR in patients presenting with seven days of fever (Figure 6.4).



(A)*



(B)



(C)

Figure 6.3 Effect of fever duration at presentation on tests sensitivity for early diagnosis of acute scrub typhus infection (n=810).

(A) 47kDa qPCR, (B) IgM ICT, and (C) combination of 47kDa qPCR and IgM ICT

*No positive 47kDa qPCR in patients presenting with one and five days of fever

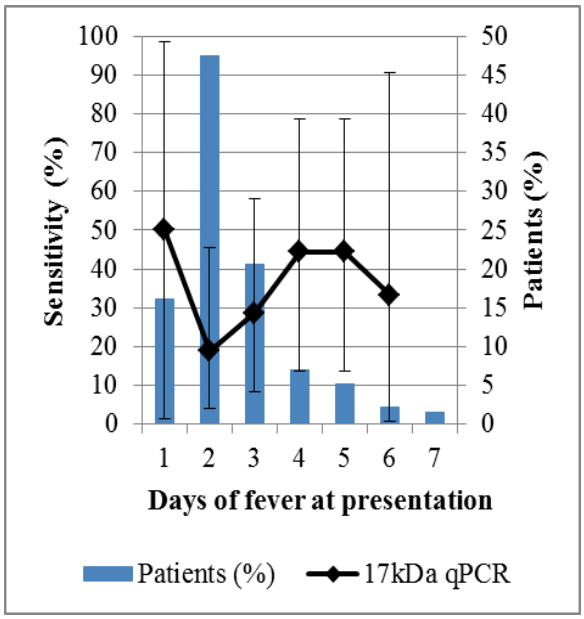


Figure 6.4 Effect of fever duration at presentation on the sensitivity of the 17kDa qPCR assay for early diagnosis of acute murine typhus infection (n=811).

Note: there were no positive 17kDa qPCR in patients presenting with seven days of fever

6.3.10 Impact of malaria infection on the diagnostic accuracy of the tests for early diagnosis of acute scrub typhus infection

For the scrub typhus diagnostic tests, 810 non-malaria febrile patients were included in the current analysis. Malarial DNA was detected in 139 patients by 18S *rRNA* qPCR assay (sub-microscopic malaria infection). The malarial DNA was detected in 22.7% (10/44) of patients with laboratory confirmed diagnosis of acute scrub typhus infection and was detected in 16.8% (129/766) of patients with laboratory confirmed non-acute scrub typhus infection by the reference tests.

The impact of malaria infection on the performance characteristics of the diagnostic tests under evaluation for early diagnosis of acute scrub typhus infection was assessed by stratified diagnostic accuracies of the tests in malaria qPCR positive patients (Table 6.8) and malaria qPCR negative patients (Table 6.9). The sensitivity of 47kDa qPCR assay was higher in malaria positive patients (30.0%) compared to the negative patients (20.6%),

however the number of true positive and false negative in malaria positive patients were small and the 95% CI around the sensitivity were wide. These differences were not statistically significant. The sensitivity of the IgM ICT and the combination of 47kDa qPCR+IgM ICT was lower in malaria positive patients (20.0 and 30.0% for IgM ICT and 47kDa qPCR+IgM ICT, respectively) compared to the negative patients (29.4 and 38.2% for IgM ICT and 47kDa qPCR+IgM ICT, respectively). They were also not significantly different.

Similarly, the specificity of 47kDa qPCR was higher in malaria positive patients (100 vs. 99.5%) and the specificity of the IgM ICT and the combination of 47kDa qPCR+IgM ICT was lower in malaria positive patients (88.4 vs. 94.5% and 88.4 vs. 94.2% for IgM ICT and 47kDa qPCR+IgM ICT, respectively). These differences were not statistically significant. There was not enough evidence to prove that malaria infection had a significant impact on the diagnostic accuracies of the tests under this evaluation. In addition, the overall clinical sensitivity of the 47kDa qPCR (22.7%), IgM ICT (27.3%) and the combination of 47kDa qPCR+IgM ICT (36.4%) was generally poor (Table 6.5) and they were not much different to the sensitivity of both malaria positive and negative patients when stratified.

Table 6.8 Impact of malaria infection on the performance characteristics of the diagnostic tests for early diagnosis of acute scrub typhus infection in malaria qPCR positive patients (n=139).

Tests		Reference results*		%Sensitivity (95% CI)	%Specificity (95% CI)	%PPV (95% CI)	%NPV (95% CI)	AUC
		Scrub typhus	Not Scrub typhus					
47kDa qPCR	+	3	0	30.0 (6.7-65.2)	100 (97.2-100)	100 (29.2-100)	94.9 (89.7-97.9)	0.6500
	-	7	129					
IgM ICT	+	2	15	20.0 (2.5-55.6)	88.4 (81.5-93.3)	11.8 (1.5-36.4)	93.4 (87.5-97.1)	0.5419
	-	8	114					
47kDa qPCR+IgM ICT	+	3	15	30.0 (6.67-65.2)	88.4 (81.5-93.3)	16.7 (3.6-41.4)	94.2 (88.4-97.6)	0.5919
	-	7	114					

47kDa qPCR= quantitative real-time PCR targeting 47kDa, AUC= area under ROC curve, CI= confidence interval, IgM ICT= IgM antibody detection immunochromatographic test, NPV= negative predictive value, PPV= positive predictive value

*IgM ELISA/IFA using paired acute and convalescent specimens

Table 6.9 Impact of malaria infection on the performance characteristics of the diagnostic tests for early diagnosis of acute scrub typhus infection in malaria qPCR negative patients (n=671).

Tests		Reference results*		%Sensitivity (95% CI)	%Specificity (95% CI)	%PPV (95% CI)	%NPV (95% CI)	AUC
		Scrub typhus	Not Scrub typhus					
47kDa qPCR	+	7	3	20.6 (8.7-37.9)	99.5 (98.6-99.9)	70.0 (34.8-93.3)	95.9 (94.1-97.3)	0.6006
	-	27	634					
IgM ICT	+	10	35	29.4 (15.1-47.5)	94.5 (92.4-96.1)	22.2 (11.2-37.1)	96.2 (94.3-97.5)	0.6196
	-	24	602					
47kDa qPCR+IgM ICT	+	13	37	38.2 (22.2-56.4)	94.2 (92.1-95.9)	26.0 (14.6-40.3)	96.6 (94.9-97.9)	0.6621
	-	21	600					

47kDa qPCR= quantitative real-time PCR targeting 47kDa, AUC= area under ROC curve, CI= confidence interval, IgM ICT= IgM antibody detection immunochromatographic test, NPV= negative predictive value, PPV= positive predictive value

*IgM ELISA/IFA using paired acute and convalescent specimens

6.3.11 Impact of malaria infection on the diagnostic accuracy of the 17kDa qPCR assay for early diagnosis of acute murine typhus infection

For the murine typhus diagnostic test, 811 non-malaria febrile patients were included in the current analysis. Of the 139 patients with sub-microscopic malaria infection, malarial DNA was detected in 3.7% (2/54) of patients with laboratory confirmed diagnosis of acute murine typhus infection and was detected in 18.1% (137/757) of patients with laboratory confirmed non-acute murine typhus infection by the reference tests. The impact of malaria infection on the performance characteristics of the 17kDa qPCR assay for early diagnosis of acute murine typhus infection was assessed by stratified diagnostic accuracies of the test in malaria qPCR positive patients (Table 6.10) and malaria qPCR negative patients (Table 6.11). The sensitivity of 17kDa qPCR assay was higher in the malaria positive patients (50.0%) compared to the negative patients (30.8%), however the number of patients with positive test in malaria positive group was small and the 95% confidence interval around the sensitivity was very wide. The specificity was similar between the malaria positive (99.3%) and negative (99.8%) patients which was very high. Similar to the performance of the scrub typhus diagnostic tests, the sensitivity of the 17kDa qPCR assay was generally poor (31.5%, Table 6.7) and not clinically useful.

Table 6.10 Impact of malaria infection on the performance characteristics of the 17kDa qPCR assay for early diagnosis of acute murine typhus infection in malaria qPCR positive patients (n=139).

Test		Reference results[b]		%Sensitivity	%Specificity	%PPV	%NPV	AUC
		Murine typhus	Not Murine typhus	(95% CI)	(95% CI)	(95% CI)	(95% CI)	
17kDa qPCR[a]	+	1	1	50.0 (1.3-98.7)	99.3 (96.0-100)	50.0 (1.3-98.7)	99.3 (96.0-100)	0.7464
	-	1	136					

17kDa qPCR= quantitative real-time PCR targeting 17kDa, AUC= area under ROC curve, CI= confidence interval, NPV= negative predictive value, PPV= positive predictive value

[a]Specimens positive for 17kDa and *ompB* qPCR assays/sequencing confirmed *R. typhi*

[b]IgM ELISA/IFA using paired acute and convalescent specimens

Table 6.11 Impact of malaria infection on the performance characteristics of the 17kDa qPCR assay for early diagnosis of acute murine typhus infection in malaria qPCR negative patients (n=672).

Test		Reference results[b]		%Sensitivity	%Specificity	%PPV	%NPV	AUC
		Murine typhus	Not Murine typhus	(95% CI)	(95% CI)	(95% CI)	(95% CI)	
17kDa qPCR[a]	+	16	1	30.8 (18.7-45.1)	99.8 (99.1-100)	94.1 (71.3-99.9)	94.5 (92.5-96.1)	0.6530
	-	36	619					

17kDa qPCR= quantitative real-time PCR targeting 17kDa, AUC= area under ROC curve, CI= confidence interval, NPV= negative predictive value, PPV= positive predictive value

[a]Specimens positive for 17kDa and *ompB* qPCR assays/sequencing confirmed *R. typhi*

[b]IgM ELISA/IFA using paired acute and convalescent specimens

6.4 Discussion

Rapid and accurate diagnostic tests are urgently needed for the diagnosis of rickettsial infections, especially in endemic areas where the infections are potentially serious and require specific treatment but cannot be reliably distinguished from other causes of fever, potentially resulting in incorrect treatment and under-reporting [172, 294]. Without laboratory diagnostic tests, it is almost impossible to differentiate scrub typhus and murine typhus infections from the other causes of fever based on the clinical presentation alone as shown in the current study where none of the febrile patients were clinically diagnosed with murine typhus infection and only one patient was clinically diagnosed with scrub typhus infection.

In the current study, the performance characteristics and clinical usefulness of an IgM ICT and two real-time qPCR assays were evaluated for the early diagnosis of scrub typhus (IgM ICT and 47kDa qPCR) and murine typhus (17kDa qPCR) infections during acute phase of febrile illness. For the scrub typhus IgM ICT, the overall performance was poor with a clinical sensitivity of 27.3%. Although the specificity was high (93.5%), the PPV and NPV were 19.4 and 95.7%, respectively. This would result in many misdiagnosed cases if the test was to be used alone. The low sensitivity of the IgM ICT could be due to the different antigens of *O. tsutsugamushi* used in the test compared to the reference tests (IgM ELISA/IFA) [229]. The Karp and Gilliam strains are predominant in Thailand [295] and the Karp, Gilliam and Kato strains were used as antigens for the reference tests in this study whereas the Boryong strain was used for the IgM ICT.

Duration of fever at presentation had an impact on the IgM ICT in this evaluation, since most patients presented very early in the course of their fever (median of two days [IQR 2-3]) when the IgM antibody had not reached the detectable level [189]. This was also true when looking at the IgM antibody titres in acute specimens tested by the IgM IFA

assay. Most of the patients with confirmed acute scrub typhus infection had low IgM IFA titres (84.1%) which were <1:400 [206] and had ≥4-fold increased titres in convalescent specimens which were collected two weeks later.

Both real-time qPCR assays were previously shown to have excellent laboratory specificity and sensitivity (three copies/µl for both assays) [210, 217, 223]. Following the validation of the assays with the ABI 7500 FAST real-time PCR system (Applied Biosystems), the assays also retained excellent laboratory sensitivity and specificity. They were capable of detecting as little as 1-10 copies of target template per reaction (1-10 copies/µl of DNA extract). The diagnostic accuracy and clinical utility of using these qPCR assays were then evaluated using acute specimens. Despite the great laboratory sensitivity and specificity of the assays during validation that could potentially be used for diagnosis, the clinical sensitivity was disappointing, with 22.7 and 31.5% for 47kDa and 17kDa assays, respectively. However, excellent clinical specificities were obtained from both assays (99.6 and 99.7% for 47kDa and 17kDa qPCRs, respectively). There are several explanations for this. Firstly, the low clinical diagnostic sensitivity was attributed to low bacterial loads. The median *O. tsutsugamushi* bacterial loads from 30.8% (4/13) quantifiable positive specimens were 905 copies/ml of blood (range 600-1,280). This corresponded to 18 copies/reaction (range 12.0-25.6). The quantitative data suggested that the bacterial loads in blood during rickettsial illness were too low to be reliably detectable using molecular methods. In particular, the number of positive results below the accurate quantification level obtained from both assays is indicative of analyte levels below the threshold of the assay accurate detection (<10 copies/reaction). At such quantities, they are not always reliably detectable as defined by the MIQE guideline [296]. The molecular diagnostic challenges for scrub typhus and murine typhus infections with low bacterial loads have been described elsewhere and the data suggested that *O. tsutsugamushi* bacterial loads were approximately 10-fold higher than *R. typhi* bacterial loads in patients

from Thailand and Laos [230]. This is in line with the results in the present study where the *O. tsutsugamushi* bacterial loads seem to be higher as 30.8% (4/13) of them were quantifiable, although the number was small. To improve the assay detection limit, increasing the specimen volume can be performed, however it requires an optimisation as it also increases the human genomic DNA that could have a negative impact on the performance of the test and it might not be feasible in some clinical settings. Molecular methods generally have an advantage over serological methods as they are able to detect bacterial DNA during early rickettsial illness when the antibody is rarely detectable [189]. However, they did not show much advantage in the present study, since the overall sensitivity of the qPCR assays was low and there were insufficient data when stratified by duration of fever at presentation.

Secondly, laboratory confirmation in this evaluation was based on the results of gold standard paired serology using IgM ELISA/IFA assays, of which IFA is known to be an imperfect gold standard. Interpretation of IFA results is inherently subjective and it is difficult to standardise due to various, or lack of, local cut-offs [191, 202]. This may be affecting the diagnostic accuracy of the tests under evaluation, however, the conservative four-fold rising titres between paired acute and convalescent specimens were used in the current evaluation. The recent robust Scrub Typhus Infection Criteria (STIC; a combination of culture, IgM IFA, and PCR assays) was reported, which provided a high confidence in confirmed diagnosis and was proposed to be used as a reference for evaluating alternative diagnostic tests [228, 229]. However, it was not possible to fully implement in the current evaluation, since BSL-3 facilities were not available at SMRU for performing culture of the organisms and only one PCR assay was available which was under evaluation. Moreover, the low specificity of the STIC due to the low specificity of the IgM IFA was reported with Bayesian LCMs, the most recent analysis method to estimate the true accuracy of diagnostic tests where the gold standard is imperfect [195].

The impact of malaria infection on the performance characteristics of the diagnostic tests was assessed. Both qPCR assays and the IgM ICT remained highly specific and poorly sensitive independent of malaria infection status.

In summary, despite the excellent specificity of the IgM ICT, 47kDa and 17kDa qPCR assays, all of them had poor sensitivity and were not appropriate to use as an early diagnostic tool for scrub typhus and murine typhus infections. This study highlights the need for rapid and accurate early diagnostic tools for scrub typhus and murine typhus infections.

7 Implementation of a leptospirosis qPCR assay

7.1 Introduction and aim

Laboratory diagnosis is important to confirm leptospirosis because clinical diagnosis is difficult and inaccurate due to its broad range and non-specific clinical presentations. The diagnosis of leptospirosis by the gold standard serology, MAT, or culture requires an experienced laboratory. These assays are time consuming, laborious and available at the reference laboratories, resulting in diagnosis delays. RDTs are easy to use as rapid bedside tests, but lack accuracy for early diagnosis of acute infection [146]. Detection of nucleic acid, for example by PCR, is useful during the acute phase of infection. This chapter describes the validation and implementation of the leptospirosis qPCR assay. Since there were no leptospirosis diagnostic tests available at SMRU at the time of the study, the aims were to introduce, validate and implement the 16S *rRNA* qPCR assay for detection of *Leptospira* spp. DNA in acute phase specimens for early diagnosis of leptospirosis.

7.2 Materials and Methods

7.2.1 Patient specimens and methods

Acute plasma specimens from 908 non-malaria febrile patients were tested using the 16S *rRNA* qPCR assay to detect the *Leptospira* DNA for early diagnosis of acute leptospirosis infection.

The 16S *rRNA* qPCR assay was validated at SMRU laboratory using ABI 7500 FAST real-time PCR system (Applied Biosystems). The LoD of the assay was assessed to determine the laboratory sensitivity of the assay for the detection of *Leptospira* DNA. A 10-fold serial dilution of the linearised plasmid DNA standard control from *L. interrogans* starting from 10,000 to 1 copies/µl was constructed and tested in duplicate. The assay

validation was performed for 20 replicates (each 10-fold serial dilution was tested in duplicate for 10 qPCR runs). An average R^2 value (coefficient of correlation) obtained for the standard curve and efficiency of the qPCR assay were reported. Specificity of the assay was determined by testing with 1.0 ng/µl of the following organisms: *E. faecalis*, *E. coli*, *P. falciparum*, *P. vivax*, *Ps. aeruginosa*, *S. aureus*, *Streptococcus* Group B and *S. pneumoniae*, and 3.3-33.3 PFU/µl of DENV1-4 mix.

Bacterial loads were calculated with the following formula: number of the target DNA copies/ml of plasma = [(number of copies/µl of DNA template)/2] x 1,000. Number of copies/µl of DNA template were calculated using 10-fold serial dilution of the linearised plasmid DNA (10,000-1 copies/µl), resulting in numbers of copies/µl of DNA extract. The factor 2 adjusted for the 1:2 ratio of DNA extract to plasma, resulting in number of copies/µl of plasma. The factor 1,000 corrected for the copies/µl of plasma to copies/ml of plasma. The *Leptospira* spp. have two copies of the 16S *rRNA* gene [150], therefore the number of copies/ml of plasma is divided by 2 resulting in the number of genomic equivalent (GE)/ml of plasma.

7.2.2 Data analysis

Data were analysed using STATA/SE 10.1 (StataCorp LP). The agreement between clinical diagnosis and laboratory diagnosis of acute leptospirosis infection was calculated using Kappa statistics [286].

7.3 Results

7.3.1 Laboratory sensitivity and specificity of 16S *rRNA* qPCR assay

The laboratory sensitivity of the 16S *rRNA* qPCR assay was determined to be 1 copy/µl or 0.5 GE/µl of DNA template (equivalent to 500 copies/ml or 250 GE/ml of plasma). All 20 replicates were positive for each dilution point (100% repeatability and reproducibility). Following the validation of the assay, an optimal fluorescence threshold

was set at 10,000 for all qPCR runs. Standard curves were generated from the amplification plots. The average R^2 value of the standard curves was 1.00 and the assay efficiency was 95.7%. Figure 7.1 shows examples of amplification curve and standard curve for a qPCR run of the assay. For the laboratory specificity, this assay was specific as there were no amplifications for all other organisms tested using this cut-off threshold.

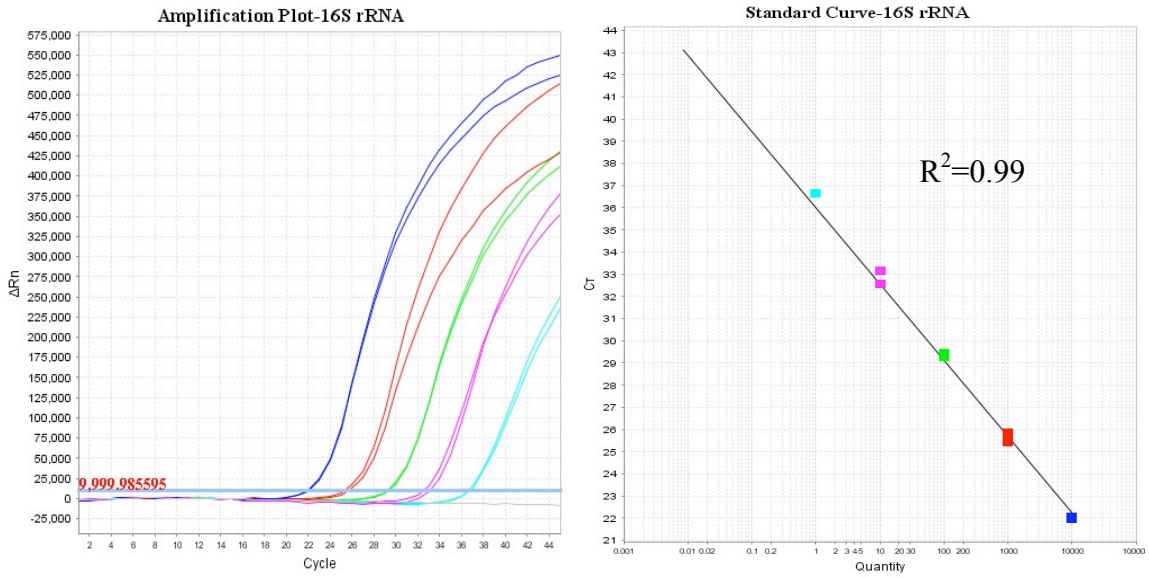


Figure 7.1 Amplification curve and standard curve analysis of the 16S *rRNA* qPCR for the detection of *Leptospira* DNA.

Legend: blue=10,000 copies/µl, red=1,000 copies/µl, green=100 copies/µl, pink=10 copies/µl, light blue=1 copy/µl, and grey=negative control.

7.3.2 Detection of *Leptospira* DNA by 16S *rRNA* qPCR assay

Acute plasma specimens from 908 febrile patients were tested using the 16S *rRNA* qPCR assay for the detection of *Leptospira* DNA. Of these, 7.5% (68/908) of the patients were given a positive result. Quantification of the *Leptospira* DNA was obtained from 68 positive plasma specimens. The copy number ranged from 1.0 to 297.4 copies/µl of DNA extract with a median of 6.9 copies/µl of DNA extract (IQR 3.4-18.6), equivalent to 250 to 74,350 GE/ml of plasma with a median of 1,725 GE/ml of plasma (IQR 850-4,650).

Presumptive diagnoses were given at the clinics based on clinical presentations. Of the 908 patients, 20.2% (183/908) were clinically diagnosed with leptospirosis infection. The agreement between clinical diagnosis and the laboratory diagnosis for acute leptospirosis infection was poor (*Kappa* = 0.0602) (Table 7.1). The clinical diagnoses for patients with the laboratory diagnosis of acute leptospirosis are shown in Table 7.2. Of the 68 patients with the laboratory diagnosis of acute leptospirosis infection, 41.2% (28/68) of the patients were clinically diagnosed with leptospirosis at presentation.

Table 7.1 Clinical diagnosis and laboratory diagnosis of acute leptospirosis infection.

Clinical diagnosis	Laboratory diagnosis		Total
	Leptospirosis	Not leptospirosis	
Leptospirosis (%)	28 (41.2)	155 (18.5)	**183 (20.2)**
Not leptospirosis (%)	40 (58.8)	685 (81.5)	**725 (79.8)**
Total (%)	**68 (100)**	**840 (100)**	**908 (100)**

Table 7.2 Clinical diagnosis for patients with the laboratory diagnosis of acute leptospirosis infection.

Clinical diagnosis	Laboratory diagnosis of acute leptospirosis cases (%)
Leptospirosis	28 (41.2)
Unknown fever	27 (39.7)
Dengue	6 (8.8)
Scrub typhus	4 (5.9)
Typhoid	2 (2.9)
Sepsis	1 (1.5)
Total	**68 (100)**

7.4 Discussion

Clinical presentations of leptospirosis are often confused with other causes of fever, as shown in the present study where only 15.3% (28/183) of patients who were clinically diagnosed with leptospirosis had laboratory diagnosis of acute leptospirosis infection. It is therefore important to confirm diagnosis of this infection by the laboratory.

This chapter describes the validation and implementation of the 16S *rRNA* qPCR assay for leptospirosis diagnosis. The 16S *rRNA* qPCR assay for the detection of *Leptospira* spp. DNA was evaluated and used instead of the gold standard serology, MAT, as it was not available at SMRU at the time of the study. From this evaluation, the assay was found to be both sensitive and specific when using control nucleic acids. The analytical sensitivity of the assay was 1 copy/µl or 0.5 GE/µl of DNA template. The sensitivity was similar to the previously published paper from MORU where the analytical sensitivity was 10 GE/reaction or equivalent to 2 GE/µl of DNA template [149]. The specimen type, specimen volume and extraction method used in this study were different from the study published by MORU. In the present study, 200µl of plasma specimen was extracted using the MagCore HF16 automated magnetic platform (RBC Bioscience), and elution volume was 100µl, whereas at MORU 5ml of whole blood specimen was extracted using the Nucleon BACC Genomic DNA extraction kit (GE Healthcare Bioscience, Marlborough, MA, USA), and elution volume was 1ml. As these factors could have affected the clinical diagnostic accuracy of the test, further study to evaluate their impact is warranted. Although, the concentration factor between the method of MORU (1:5) and this study (1:2) was not so different.

8 Practical implications in the absence of the gold standard diagnostic tests

8.1 Introduction and aims

Differential diagnosis for non-malaria causes of AUFI is challenging in clinical settings where laboratory diagnostic tests are inadequate. Current gold standard diagnostic tests, usually based on culture of organisms or paired serology, are unsuitable for clinical use as they may take days to weeks for results. Thus, they are unable to provide results quickly enough to aid patient management [120, 191]. There is an urgent requirement for accurate and rapid diagnostic tests to identify the causes of non-malaria AUFI during the acute phase of infection. This chapter describes the practical implications of using non-gold standard diagnostic tests (referred to here as alternative tests) that are available in the SMRU setting to determine the causes of non-malaria AUFI in the absence of the gold standard diagnostic tests. The aim was to determine the causes of non-malaria AUFI in patients presenting with fever where a definitive diagnosis cannot be made in the field with a clinical useful turnaround time.

8.2 Materials and Methods

8.2.1 Patient specimens and methods

Acute blood specimens from 908 non-malaria febrile patients were tested using alternative diagnostic tests as shown in Table 8.1**Error! Reference source not found.**. Plasma specimen was used to perform rRT-PCR and ICT assays for diagnosis of acute dengue virus infection, 16S *rRNA* qPCR assay for acute leptospirosis infection and ICT for acute scrub typhus infection. Buffy coat specimen was used to perform 47kDa, 17kDa, *ompB* qPCRs and sequencing for acute rickettsial infections. Whole blood was used to perform blood culture for invasive bacterial infection.

In addition to the diagnosis of acute dengue virus infection by the alternative diagnostic tests as described above, the serotype of dengue virus was identified using a nested-RT PCR assay [74, 80]. This assay was performed using RNA extracts from all positive specimens by the dengue rRT-PCR assay. The details of all laboratory methods and their interpretations are described in the Materials and Methods chapter, section 2.9.

Table 8.1 Alternative diagnostic tests used for testing acute blood specimens.

Diseases	**Diagnostic tests**
Dengue	Dengue group specific one-step SYBR Green based rRT-PCR [16, 259], and SD BIOLINE Dengue Duo NS1, IgM/IgG ICT (Standard diagnostic, Inc.)[a]
Leptospirosis	16S *rRNA* (*Leptospira* spp.) qPCR [147, 149, 150]
Scrub typhus	47kDa qPCR [210], and SD BIOLINE Scrub typhus IgM ICT (Standard diagnostic, Inc.) [b]
Murine typhus and other *Rickettsia* spp.[c]	17kDa qPCR, *ompB* qPCR [217, 218], and Sequencing (Macrogen)
Invasive bacterial infections	BacT/ALERT blood culture (BioMérieux)

[a]Analysed using the results of NS1 and IgM for the SD BIOLINE Dengue Duo, [b]SD BIOLINE Scrub typhus IgM ICT was available for 898 tests, [c]Sequencing was performed on 17kDa positive/*ompB* negative qPCR specimens to confirm *Rickettsia* spp.

8.2.2 Data analysis

Data were analysed using STATA/SE 10.1 (StataCorp LP) and graphs were created using Microsoft Excel 2010 (Microsoft). Positive results of each alternative diagnostic test were combined using "OR" operator to determine the proportion of patients for each diagnosis.

8.3 Results

8.3.1 Diagnosis by alternative tests

All available alternative diagnostic tests in the SMRU setting were used and included in the current analysis for early diagnosis of acute infection on acute phase of specimens (Table 8.1). Regardless of the results of gold standard diagnostic tests for dengue, leptospirosis and rickettsial infections, of the 908 non-malaria febrile patients, 20.2% (183/908) of the patients were diagnosed with dengue, followed by 6.8% (62/908) for scrub typhus, 6.5% (59/908) for leptospirosis, and 1.9% (17/908) for murine typhus. The proportions of other diagnoses and co-infections were small (4.0%, 36/908), leaving 60.7% (551/908) of the patients with no diagnosis for their fever (Figure 8.1). The details of the other diagnoses and co-infections are shown in Table 8.2.

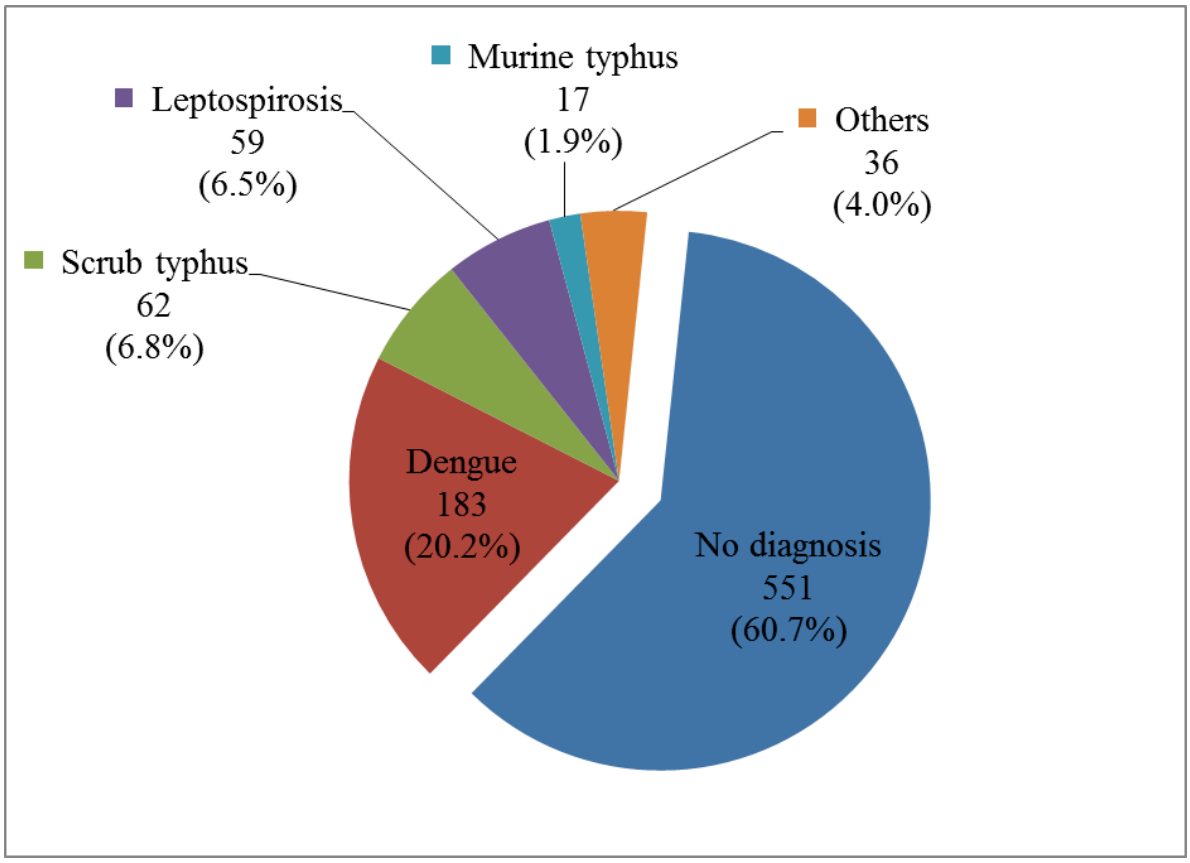


Figure 8.1 Diagnosis of 908 non-malaria febrile patients based on the results of alternative diagnostic tests.

Table 8.2 Other diagnoses and co-infections based on the results of alternative diagnostic tests.

Diagnoses	Number of patients (%)
Invasive bacterial infection*	11 (1.2)
Dengue/ Scrub typhus	10 (1.1)
Leptospirosis/ Scrub typhus	6 (0.7)
Dengue/ Murine typhus	3 (0.3)
Leptospirosis/ Dengue	3 (0.3)
Escherichia coli/ Scrub typhus	1 (0.1)
Rickettsia felis	1 (0.1)
Scrub typhus/ Murine typhus	1 (0.1)
Total	**36 (4.0)**

*As described in the chapter 4

The number of patients that were positive for each alternative diagnostic test is shown in Table 8.3. From a total of 199 dengue positive patients by rRT-PCR and/or RDT, 166 patients were positive by the rRT-PCR assay. These patients were investigated further for the infecting serotype of dengue virus. The serotype of dengue virus was identified from all 166 patients: 150 patients had DENV-1, 11 patients had DENV-3 and 5 patients had DENV-4. None of the patients were found to have DENV-2.

Table 8.3 Number of patients positive by each alternative diagnostic tests.

Diagnostic tests	Number of patients (%), n=908
Dengue	
• rRT-PCR	166 (18.3)
• NS1 ICT	144 (15.9)
• IgM ICT	43 (4.7)
• rRT-PCR+NS1+IgM	199 (21.9)
Leptospirosis	
• 16S *rRNA* qPCR	68 (7.5)
Scrub typhus	
• 47kDa qPCR	18 (2.0)
• IgM ICT[a]	73 (8.1)
• 47kDa qPCR+ IgM ICT	80 (8.8)
Murine typhus	
• 17kDa qPCR[b]	21(2.3)
Rickettsia felis	
• Sequencing	1 (N/A)[c]
Invasive bacterial infection[d]	
• Blood culture	12 (1.3)

[a]Data available for 898/908 patients, [b]Specimens positive for 17kDa and *ompB* qPCR assays/sequencing confirmed *Rickettsia typhi*, [c]Only performed to confirm this case by sequencing, [d]As described in chapter 4.

8.4 Discussion

There is a pressing need for rapid and accurate diagnostic tests to improve the diagnosis of non-malaria AUFI. The gold standard tests, either culture of the organisms or paired serology, for confirmation of dengue, leptospirosis and rickettsial infections which are the most common causes of AUFI, are not clinically useful [33, 110, 120, 196]. The results turnaround time, particularly of paired serology, is retrospective rather than prospective by definition.

This chapter describes the practical implications of using alternative diagnostic tests that are available in the SMRU setting and that have quick turnaround times to determine the causes of AUFI in a single acute phase specimen. In the absence of the gold standard diagnostic tests, diagnoses were made in 39.3% of the non-malaria febrile patients in which the proportion was slightly higher than those diagnosed by the gold standard tests (34.6%) (see chapter 4). In addition to more patients being given diagnoses based on the results of the alternative tests, the proportion of co-infections also increased compared with gold standard methods. This may be due to differences in detection windows and/or the diagnostic accuracy of the tests.

SMRU clinics provide healthcare to marginalised populations in a rural setting. The detection and treatment of malaria was one of SMRU's main goals, however, due to successful management the number of malaria cases seen has decreased drastically in recent years (Figure 1.22). Accessible diagnostic tools, such as microscopy and RDT, and expertise have been key for successful management of malaria patients. Despite the drop in the number of malaria cases, patients were still presenting at SMRU clinics with fever, however, prior to the work presented in this thesis there were no diagnostic tools to guide the management of these patients. Although it was not possible to implement gold standard serology tests at the SMRU laboratories for any of the non-malaria febrile illnesses, rapid

diagnostic tests (ICT) for dengue and scrub typhus were introduced and evaluated as well as PCR assays for dengue, leptospirosis and rickettsial infections. The performance of these diagnostic tests was evaluated and described in previous chapters of this thesis. Although not routinely available at SMRU, paired acute and convalescent serum specimens were also sent out to undergo the reference serology tests for dengue (at AFRIMS), and scrub typhus and murine typhus (at MORU) for confirmation of infections. Even though the follow-up rate was high in the present study, the convalescent serum specimen was not available in 10.6% (96/908) of the patients that were lost follow-up. The lack of a convalescent specimen is the main obstacle in diagnosis of acute infection based on the gold standard paired serology [191]. The accuracy of the serology tests varies based on the disease prevalence, clinical variability, and availability and timing of the convalescent specimen [297]. Although, the evaluation of the optimal cut-off point and accuracy of the serology tests have been described, further studies to evaluate these in different settings are still required [192, 195, 207]. In the present study, patients with only a single specimen were not analysed by the reference serology but all acute blood specimens were tested and analysed using alternative diagnostic tests. Of these single specimens, 10/96 patients had diagnosis of leptospirosis by the 16S *rRNA* qPCR assay.

There are several points that need to be addressed in the practical selection of alternative diagnostic tests used for early diagnosis of acute infection in the SMRU setting. The clinical diagnostic accuracy needs to be evaluated in the setting where the tests are being implemented. The clinical diagnostic accuracy of a test may differ from the analytical accuracy in the controlled laboratory environment (chapter 5-6) or in different clinical settings. This is in relation to the detection window of the tests and the timing of specimen collection. For example, for molecular diagnostic tools for detection of nucleic acid of the causative agent during viraemia or bacteraemia, the specimen should be collected during the acute phase of infection or soon after the symptom onset. On the other

hand, antibody based detection might not be useful during this time since the detectable antibody may take several days to weeks to develop. Specific antibody is rarely detectable in acute specimens, especially when patients present early in the course of their illness as was the case for this study (a median of two days (IQR 2-3) duration of fever). The result turnaround time, cost, and availability of the tests all need to be considered. The diagnostic tests should ideally have rapid result turnaround times so that useful to assist patient management. The cost of the tests should be affordable, especially in resource poor settings where AUFI is common. There should be a reliable and affordable supply chain to the site where they are needed. The estimated costs and turnaround times for the alternative diagnostic tests used in this study are shown in Appendix 3. In addition, other considerations include storage conditions, shelf life of the product, skills required to perform the test and requirement for additional equipment.

In resource limited settings, the diagnostic test of choice should be considered according to the WHO ASSURED criteria (Affordable, Sensitive, Specific, User friendly, Robust and rapid, Equipment free, Deliverable to those who need them) [298]. Only few accessible rapid diagnostic tests for non-malaria AFUI met these criteria, albeit with limited sensitivity and specificity [15]. The present study has shown that the new generation rapid test that included NS1 antigen detection for early diagnosis of acute dengue infection is clinically useful for the management of patients and fit with the ASSURED model (chapter 5). For scrub typhus, the IgM ICT had high specificity but the sensitivity was poor. It was not found to be appropriate to use as an early diagnostic test in the field. The test is also not yet commercially available (chapter 6). The molecular methods have high sensitivity and specificity and could be able to detect non-cultivable or slow growing pathogens to get quicker results than the culture of these pathogens, but generally failed to meet the ASSURED criteria in terms of affordable, equipment free, rapid and not easy to deliver to use in the field [299]. As shown in the present study, the

dengue rRT-PCR assay had excellent performance for early diagnosis using a single specimen but did not fulfil the ASSURED criteria (chapter 5). For scrub typhus and murine typhus, the performance of the qPCR assays was not good enough to use as a single diagnostic test since the sensitivity of the tests were poor, although the specificity was high (chapter 6). For leptospirosis, the analytical sensitivity and specificity of the 16S *rRNA* qPCR were excellent. An evaluation of clinical diagnostic accuracy is required in the current setting, although the evaluation has been done elsewhere [149].

There is no single diagnostic test that is perfect and clinically useful in the resource limited setting that meet the ASSURED criteria. Different diagnostic tests each have their own limitations. The clinical diagnostic accuracy of the tests, optimal detection window, timing of specimen collection, result turnaround time, cost, and availability of the tests need to be considered to determine the optimal testing strategy for early diagnosis of acute infection to assist the management of the non-malaria AUFI patients. It is difficult to find an ASSURED based diagnostic test that is comparable to the optimal laboratory based assays. However, a slightly less sensitive rapid bedside test might be more useful to patients as it could provide the result in time to guide the correct treatment. If budget is available, the rRT-PCR for dengue could be used as the reference test in the absence of gold standard serology. This would be appropriate to the current setting of the SMRU where the molecular laboratory is already in place, although this might not be feasible in other settings.

This study suggests the SD BIOLINE Dengue Duo NS1, IgM/IgG ICT (Standard diagnostic, Inc.) is suitable to implement in the resource limited clinical setting. The other alternative diagnostic tests under evaluation for leptospirosis and rickettsial infections are not recommended.

9 Concluding remarks

The work described in this thesis has determined the diagnostic accuracy and clinical value of using rapid tests to diagnose causes of non-malaria fever focussing on dengue, leptospirosis, and rickettsial infections (mainly scrub typhus and murine typhus) in a rural SE Asian clinical setting.

9.1 Key findings

1. Using the reference laboratory diagnostic tests to confirm the causes of non-malaria AUFI in this study, a diagnosis was confirmed in 34.6% of the patients. Dengue was the most common diagnosis (15.9%), followed by leptospirosis (6%), murine typhus (6%), and scrub typhus (3.2%). Co-infection was not uncommon, occurring in 1.7% of the patients with concomitant leptospirosis and scrub typhus accounting for almost all of these cases (1.5%).

2. The new generation ICT for dengue diagnosis that included NS1 antigen detection (SD BIOLINE Dengue Duo NS1, IgM/IgG ICT, Standard diagnostic, Inc.) was useful for patient management and appropriate to implement in the field for early diagnosis of acute infection.

3. The molecular diagnostic tool, rRT-PCR, could replace the gold standard serology for early diagnosis of acute dengue infection using a single specimen.

4. Neither rapid diagnostic test (SD BIOLINE Scrub typhus IgM ICT, Standard diagnostic, Inc.) nor molecular diagnostic assays (47kDa and 17kDa qPCRs) under this evaluation were clinically useful for diagnosis of scrub typhus or murine typhus infection.

5. CRP was clinically useful to distinguish between acute viral and bacterial infections as the CRP level was more elevated in bacterial infection.

6. Malarial DNA was detected in 17.5% of non-malaria febrile patients with very low parasitaemia, which was unlikely to be the cause of their fever.

7. In malaria patients, co-infection with scrub typhus was common in this area.

9.2 General discussion

While malaria has significantly decreased globally, non-malaria causes of fever have become apparent and remains a major cause of AUFI in SE Asia. In rural and resource limited settings, diagnostic tests for non-malaria causes of AUFI are inadequate for clinical use. When patients present with non-malaria fever, it is impossible to give a definitive diagnosis based on the clinical presentation alone, and therefore without access to reliable diagnostic tests certain infections are mis- or under-diagnosed. This work was conducted to determine the optimal testing strategies for diagnosis of the common non-malaria causes of fever and improve understanding of these infections to assist management of patients in a rural SE Asia clinical setting.

When implementing new testing strategies, it is important to look at all available data relevant to the setting. Prior to this study there were only a few publications looking at the causes of fever on the Thailand-Myanmar border and in Thailand. This work is the first laboratory-based investigation in the SMRU to confirm diagnosis and describe non-malaria causes of fever in non-pregnant migrant and refugee populations. With a large cohort recruited over two years it was possible to determine the clinical diagnostic accuracy of tests with a clinically useful turnaround time. With limited resources, the work was focussed on diagnosis of dengue, leptospirosis and rickettsial infections (mainly scrub typhus and murine typhus). These infections have previously been found to be common causes of AUFI in SE Asia and existing data from SMRU also indicated that these infections were common in the SMRU target population (data obtained from SMRU pregnancy study conducted between 2004-2006 and a pilot non-pregnancy fever study

conducted in 2008). Hence, the inclusion of these diseases in the evaluation of diagnostic tests is directly useful to the local population and will be useful to the wider SE Asia population.

The diagnosis of non-malaria AUFI remains challenging. The causes include a wide variety of infections with non-specific clinical presentations. Whilst gold standard diagnostic tests for the common causes of AUFI are not available in the SMRU laboratories, even if they were the results would not be given to the clinician in time to guide patient management. In practice, diagnostic tools for dengue, leptospirosis, scrub typhus and murine typhus have not been available in SMRU. This study has validated the use of ICT and rRT-PCR for early diagnosis of acute dengue infection. This ICT is now implemented at SMRU for routine diagnosis of dengue and the rRT-PCR is available for confirmatory testing. The 16S *rRNA* qPCR for leptospirosis had high analytical performance and could potentially be useful for the early diagnosis of leptospirosis, however, further evaluation of its clinical diagnostic accuracy in this setting is required. For scrub typhus and murine typhus, diagnostic tests under this evaluation were inadequate for clinical use in routine diagnosis. Although highly specific, they were found to lack sensitivity and should therefore only be used with the knowledge that a negative result does not rule out the possibility of rickettsial disease. Development and assessment of improved diagnostic tests for these infections warrant further investigation. While composite gold standard diagnostic tests, for example a combination of culture, antigen/antibody based detection and nucleic acid detection, have been proposed and widely used to robustly confirm diagnosis for research purposes, this approach is not feasible in most routine clinical diagnostic settings due to the delay in getting results and cost effectiveness.

Many other causes of AUFI, that have not been investigated in this study, remain to be investigated and more than half of the patients remain undiagnosed. The study has built a bio-bank of well-characterised clinical specimens that could be useful for future diagnostic test evaluations, to investigate the presence of known and emerging pathogens including Zika virus and Chikunganya virus and to enable novel genomic approaches for pathogen discovery to be applied. This should enable a more complete picture of the causes of AUFI in this setting to be ascertained.

9.3 Limitations

The number of patients with confirmed diagnosis of acute primary dengue infection in this study was small. This limited the evaluation of the dengue ICT and the rRT-PCR assay in patients with acute primary dengue infection compared to the patients with acute secondary dengue infection. However, this did not compromise the clinical usefulness of the tests for this region or other dengue endemic areas including Thailand where 87% of dengue cases are secondary infections [41]. In addition, the tests tended to perform better for primary dengue infection [288, 290, 300].

The gold standard serology for confirmation of leptospirosis infection, MAT, was not available at the time of the study nor were there any other diagnostic tools available for diagnosis of leptospirosis infection at SMRU. Although MAT testing could have been performed externally, there was insufficient funding at the time of the study to cover a test that was not regarded to be accurate. Therefore, the qPCR assay that had previously been validated for human leptospirosis cases in Thailand was introduced and used [149]. The evaluation of the clinical diagnostic accuracy of this test could therefore not be determined in this study, however, archived specimens are available for future testing if and when possible.

Diagnosis of rickettsial infections is notoriously difficult. IFA, an imperfect gold standard serology test that has been used for confirmation of the scrub typhus and murine typhus infections for decades was used in the present study. There are a number of ways to interpret the results, and for this study a ≥4-fold rising titres between paired acute and convalescent specimens was applied to identify cases. The STIC interpretation criteria were not followed, as it was not feasible to implement them retrospectively [228, 229]. Bayesian LCMs, the most recent analysis method to estimate true accuracy of diagnostic tests where the gold standard is imperfect could be useful to improve the analysis in the study [195].

Although patients with a clear clinical diagnosis of a respiratory tract infection were excluded, 50% of undiagnosed cases did have a cough. Therefore, it is possible that some of the patients had a respiratory tract infection and detection of respiratory pathogens may have improved the diagnostic yield in this study. Unfortunately, appropriate specimens for respiratory tract pathogens were not collected during the study and therefore retrospective testing is not possible.

Early diagnosis of non-malaria AUFI remains challenging. The need for a rapid and accurate diagnostic test remains notably urgent. There is considerably more to be done to develop clinically useful diagnostic tests for AUFI in low- and middle-income countries.

In spite of the limitations described above, all objectives and aims that are stated in section 1.7 were addressed.

9.4 On-going and future research

Since the first large outbreak of Zika virus infection on Yap Island in the Federated States of Micronesia in 2007, Zika virus infection has become a global concern. Thailand reported cases in 2012-2014, indicating that the virus is endemic in Thailand [301]. Thailand has also recently reported the first two locally acquired cases of Zika-linked

microcephaly in SE Asia [302]. The specimens from the current study are being tested by PCR and serology assays to look for evidence of acute Zika virus infection in the population. So far, the preliminary result shows that 4/908 acute specimens are positive for Zika virus by three different PCR assays (two published assays by Lanciotti *et al.*, 2008 and one in-house assay developed by Agency for Science, Technology and Research (A* STAR, Connexis North Tower, Singapore) [303]. This is the first report of Zika virus on the Thailand-Myanmar border, with positive cases for years 2011-2012. Although, the number of positive cases detected by PCR is low, this work confirms that Zika virus is a cause of AUFI in this region. On-going work is now focusing on completing the serology analysis and current work investigating the occurrence of Zika virus in pregnancy. Further investigation for the occurrence of Chikungunya virus in the area is also underway as it shares the same vector with dengue virus and Zika virus.

Next generation sequencing technology is becoming more widely available and can be used to identify novel microbes present in clinical specimens. This genomic approach for pathogen discovery has been successfully used to discover and characterise novel viral pathogens [304]. In the present study, the causes of AUFI remain unknown in more than half of the non-malaria febrile patients and it is planned to use a sequencing based approach to investigate them further.

10 Reference

1. Archibald, L.K. and Reller, L.B., *Clinical microbiology in developing countries.* Emerg Infect Dis, 2001. **7**(2): p. 302-5.
2. Susilawati, T.N. and McBride, W.J., *Acute undifferentiated fever in Asia: a review of the literature.* Southeast Asian J Trop Med Public Health, 2014. **45**(3): p. 719-26.
3. Petersdorf, R.G. and Beeson, P.B., *Fever of unexplained origin: report on 100 cases.* Medicine (Baltimore), 1961. **40**: p. 1-30.
4. Vinetz, J.M., *Detection of leptospirosis in India.* Arch Dis Child, 2003. **88**(12): p. 1033.
5. Naing, C. and Kassim, A.I., *Scaling-up attention to nonmalaria acute undifferentiated fever.* Trans R Soc Trop Med Hyg, 2012. **106**(6): p. 331-2.
6. Leelarasamee, A., Chupaprawan, C., Chenchittikul, M., and Udompanthurat, S., *Etiologies of acute undifferentiated febrile illness in Thailand.* J Med Assoc Thai, 2004. **87**(5): p. 464-72.
7. Suttinont, C., Losuwanaluk, K., Niwatayakul, K., Hoontrakul, S., Intaranongpai, W., Silpasakorn, S., Suwancharoen, D., Panlar, P., Saisongkorh, W., Rolain, J.M., Raoult, D., and Suputtamongkol, Y., *Causes of acute, undifferentiated, febrile illness in rural Thailand: results of a prospective observational study.* Ann Trop Med Parasitol, 2006. **100**(4): p. 363-70.
8. Phuong, H.L., de Vries, P.J., Nga, T.T., Giao, P.T., Hung le, Q., Binh, T.Q., Nam, N.V., Nagelkerke, N., and Kager, P.A., *Dengue as a cause of acute undifferentiated fever in Vietnam.* BMC Infect Dis, 2006. **6**: p. 123.
9. Watt, G. and Jongsakul, K., *Acute undifferentiated fever caused by infection with Japanese encephalitis virus.* Am J Trop Med Hyg, 2003. **68**(6): p. 704-6.

10. McGready, R., Ashley, E.A., Wuthiekanun, V., Tan, S.O., Pimanpanarak, M., Viladpai-Nguen, S.J., Jesadapanpong, W., Blacksell, S.D., Peacock, S.J., Paris, D.H., Day, N.P., Singhasivanon, P., White, N.J., and Nosten, F., *Arthropod borne disease: the leading cause of fever in pregnancy on the Thai-Burmese border.* PLoS Negl Trop Dis, 2010. **4**(11): p. e888.

11. Ellis, R.D., Fukuda, M.M., McDaniel, P., Welch, K., Nisalak, A., Murray, C.K., Gray, M.R., Uthaimongkol, N., Buathong, N., Sriwichai, S., Phasuk, R., Yingyuen, K., Mathavarat, C., and Miller, R.S., *Causes of fever in adults on the Thai-Myanmar border.* Am J Trop Med Hyg, 2006. **74**(1): p. 108-13.

12. Mayxay, M., Castonguay-Vanier, J., Chansamouth, V., Dubot-Peres, A., Paris, D.H., Phetsouvanh, R., Tangkhabuanbutra, J., Douangdala, P., Inthalath, S., Souvannasing, P., Slesak, G., Tongyoo, N., Chanthongthip, A., Panyanouvong, P., Sibounheuang, B., Phommasone, K., Dohnt, M., Phonekeo, D., Hongvanthong, B., Xayadeth, S., Ketmayoon, P., Blacksell, S.D., Moore, C.E., Craig, S.B., Burns, M.A., von Sonnenburg, F., Corwin, A., de Lamballerie, X., Gonzalez, I.J., Christophel, E.M., Cawthorne, A., Bell, D., and Newton, P.N., *Causes of non-malarial fever in Laos: a prospective study.* Lancet Glob Health, 2013. **1**(1): p. e46-54.

13. White, N.J., Pukrittayakamee, S., Hien, T.T., Faiz, M.A., Mokuolu, O.A., and Dondorp, A.M., *Malaria.* Lancet, 2014. **383**(9918): p. 723-35.

14. Luxemburger, C., Nosten, F., Kyle, D.E., Kiricharoen, L., Chongsuphajaisiddhi, T., and White, N.J., *Clinical features cannot predict a diagnosis of malaria or differentiate the infecting species in children living in an area of low transmission.* Trans R Soc Trop Med Hyg, 1998. **92**(1): p. 45-9.

15. Chappuis, F., Alirol, E., d'Acremont, V., Bottieau, E., and Yansouni, C.P., *Rapid diagnostic tests for non-malarial febrile illness in the tropics.* Clin Microbiol Infect, 2013. **19**(5): p. 422-31.

16. Watthanaworawit, W., Turner, P., Turner, C.L., Tanganuchitcharnchai, A., Jarman, R.G., Blacksell, S.D., and Nosten, F.H., *A prospective evaluation of diagnostic methodologies for the acute diagnosis of dengue virus infection on the Thailand-Myanmar border.* Trans R Soc Trop Med Hyg, 2011. **105**(1): p. 32-7.

17. Syhavong, B., Rasachack, B., Smythe, L., Rolain, J.M., Roque-Afonso, A.M., Jenjaroen, K., Soukkhaserm, V., Phongmany, S., Phetsouvanh, R., Soukkhaserm, S., Thammavong, T., Mayxay, M., Blacksell, S.D., Barnes, E., Parola, P., Dussaix, E., Raoult, D., Humphreys, I., Klenerman, P., White, N.J., and Newton, P.N., *The infective causes of hepatitis and jaundice amongst hospitalised patients in Vientiane, Laos.* Trans R Soc Trop Med Hyg, 2010. **104**(7): p. 475-83.

18. Mueller, T.C., Siv, S., Khim, N., Kim, S., Fleischmann, E., Ariey, F., Buchy, P., Guillard, B., Gonzalez, I.J., Christophel, E.M., Abdur, R., von Sonnenburg, F., Bell, D., and Menard, D., *Acute undifferentiated febrile illness in rural Cambodia: a 3-year prospective observational study.* PLoS One, 2014. **9**(4): p. e95868.

19. Chheng, K., Carter, M.J., Emary, K., Chanpheaktra, N., Moore, C.E., Stoesser, N., Putchhat, H., Sona, S., Reaksmey, S., Kitsutani, P., Sar, B., van Doorn, H.R., Uyen, N.H., Van Tan, L., Paris, D.H., Blacksell, S.D., Amornchai, P., Wuthiekanun, V., Parry, C.M., Day, N.P., and Kumar, V., *A prospective study of the causes of febrile illness requiring hospitalization in children in Cambodia.* PLoS One, 2013. **8**(4): p. e60634.

20. Kasper, M.R., Blair, P.J., Touch, S., Sokhal, B., Yasuda, C.Y., Williams, M., Richards, A.L., Burgess, T.H., Wierzba, T.F., and Putnam, S.D., *Infectious*

etiologies of acute febrile illness among patients seeking health care in south-central Cambodia. Am J Trop Med Hyg, 2012. **86**(2): p. 246-53.

21. Brown, G.W., Shirai, A., Jegathesan, M., Burke, D.S., Twartz, J.C., Saunders, J.P., and Huxsoll, D.L., *Febrile illness in Malaysia--an analysis of 1,629 hospitalized patients.* Am J Trop Med Hyg, 1984. **33**(2): p. 311-5.

22. Capeding, M.R., Chua, M.N., Hadinegoro, S.R., Hussain, II, Nallusamy, R., Pitisuttithum, P., Rusmil, K., Thisyakorn, U., Thomas, S.J., Huu Tran, N., Wirawan, D.N., Yoon, I.K., Bouckenooghe, A., Hutagalung, Y., Laot, T., and Wartel, T.A., *Dengue and other common causes of acute febrile illness in Asia: an active surveillance study in children.* PLoS Negl Trop Dis, 2013. **7**(7): p. e2331.

23. Cui, L., Yan, G., Sattabongkot, J., Cao, Y., Chen, B., Chen, X., Fan, Q., Fang, Q., Jongwutiwes, S., Parker, D., Sirichaisinthop, J., Kyaw, M.P., Su, X.Z., Yang, H., Yang, Z., Wang, B., Xu, J., Zheng, B., Zhong, D., and Zhou, G., *Malaria in the Greater Mekong Subregion: heterogeneity and complexity.* Acta Trop, 2012. **121**(3): p. 227-39.

24. Guzman, M.G., Halstead, S.B., Artsob, H., Buchy, P., Farrar, J., Gubler, D.J., Hunsperger, E., Kroeger, A., Margolis, H.S., Martinez, E., Nathan, M.B., Pelegrino, J.L., Simmons, C., Yoksan, S., and Peeling, R.W., *Dengue: a continuing global threat.* Nat Rev Microbiol, 2010. **8**(12 Suppl): p. S7-16.

25. Guzman, M.G. and Kouri, G., *Advances in dengue diagnosis.* Clin Diagn Lab Immunol, 1996. **3**(6): p. 621-7.

26. Chambers, T.J., Hahn, C.S., Galler, R., and Rice, C.M., *Flavivirus genome organization, expression, and replication.* Annu Rev Microbiol, 1990. **44**: p. 649-88.

27. Weaver, S.C. and Vasilakis, N., *Molecular evolution of dengue viruses: contributions of phylogenetics to understanding the history and epidemiology of the preeminent arboviral disease.* Infect Genet Evol, 2009. **9**(4): p. 523-40.

28. Chen, R. and Vasilakis, N., *Dengue--quo tu et quo vadis?* Viruses, 2011. **3**(9): p. 1562-608.

29. Mustafa, M.S., Rasotgi, V., Jain, S., and Gupta, V., *Discovery of fifth serotype of dengue virus (DENV-5): A new public health dilemma in dengue control.* Med J Armed Forces India, 2015. **71**(1): p. 67-70.

30. Jansen, C.C. and Beebe, N.W., *The dengue vector Aedes aegypti: what comes next.* Microbes Infect, 2010. **12**(4): p. 272-9.

31. Bhattacharya, M.K., Maitra, S., Ganguly, A., Bhattacharya, A., and Sinha, A., *Dengue: a growing menace -- a snapshot of recent facts, figures & remedies.* Int J Biomed Sci, 2013. **9**(2): p. 61-7.

32. Simmons, C.P., Farrar, J.J., Nguyen v, V., and Wills, B., *Dengue.* N Engl J Med, 2012. **366**(15): p. 1423-32.

33. World Health Organization (WHO). *Dengue: guidelines for diagnosis, treatment, prevention and control*. 2009; Available from: http://www.who.int/rpc/guidelines/9789241547871/en/.

34. Guzman, M.G. and Harris, E., *Dengue.* Lancet, 2015. **385**(9966): p. 453-65.

35. World Health Organization (WHO), *Global strategy for dengue prevention and control 2012-2020.* Geneva, Switzerland: WHO, 2012.

36. Murray, N.E., Quam, M.B., and Wilder-Smith, A., *Epidemiology of dengue: past, present and future prospects.* Clin Epidemiol, 2013. **5**: p. 299-309.

37. Tantawichien, T., *Dengue fever and dengue haemorrhagic fever in adolescents and adults.* Paediatr Int Child Health, 2012. **32 Suppl 1**: p. 22-7.

38. Bhatt, S., Gething, P.W., Brady, O.J., Messina, J.P., Farlow, A.W., Moyes, C.L., Drake, J.M., Brownstein, J.S., Hoen, A.G., Sankoh, O., Myers, M.F., George, D.B., Jaenisch, T., Wint, G.R., Simmons, C.P., Scott, T.W., Farrar, J.J., and Hay, S.I., *The global distribution and burden of dengue.* Nature, 2013. **496**(7446): p. 504-7.

39. Gubler, D.J., *Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century.* Trends Microbiol, 2002. **10**(2): p. 100-3.

40. Shepard, D.S., Undurraga, E.A., and Halasa, Y.A., *Economic and disease burden of dengue in Southeast Asia.* PLoS Negl Trop Dis, 2013. **7**(2): p. e2055.

41. Guzman, A. and Isturiz, R.E., *Update on the global spread of dengue.* Int J Antimicrob Agents, 2010. **36 Suppl 1**: p. S40-2.

42. Ooi, E.E. and Gubler, D.J., *Dengue in Southeast Asia: epidemiological characteristics and strategic challenges in disease prevention.* Cad Saude Publica, 2009. **25 Suppl 1**: p. S115-24.

43. World Health Organization (WHO), *Comprehensive guidelines for prevention and control of dengue and dengue haemorrhagic fever. Revise and expanded edition.* New Delhi, India: WHO, 2011.

44. Limkittikul, K., Brett, J., and L'Azou, M., *Epidemiological trends of dengue disease in Thailand (2000-2011): a systematic literature review.* PLoS Negl Trop Dis. **8**(11): p. e3241.

45. Endy, T.P., Nisalak, A., Chunsuttiwat, S., Libraty, D.H., Green, S., Rothman, A.L., Vaughn, D.W., and Ennis, F.A., *Spatial and temporal circulation of dengue virus serotypes: a prospective study of primary school children in Kamphaeng Phet, Thailand.* Am J Epidemiol, 2002. **156**(1): p. 52-9.

46. Gubler, D.J., Suharyono, W., Sumarmo, Wulur, H., Jahja, E., and Sulianti Saroso, J., *Virological surveillance for dengue haemorrhagic fever in Indonesia using the mosquito inoculation technique.* Bull World Health Organ, 1979. **57**(6): p. 931-6.

47. Fried, J.R., Gibbons, R.V., Kalayanarooj, S., Thomas, S.J., Srikiatkhachorn, A., Yoon, I.K., Jarman, R.G., Green, S., Rothman, A.L., and Cummings, D.A.T., *Serotype-Specific Differences in the Risk of Dengue Hemorrhagic Fever: An Analysis of Data Collected in Bangkok, Thailand from 1994 to 2006.* PLoS Negl Trop Dis, 2010. **4**(3).

48. Ministry of Public Health. *Summary of notifiable diseases: reported number and rate per 100,000 population of cases and deaths by province and year, Thailand, 2003-2014*. 2014 (Accessed 20 September 2015)]; Available from: http://www.boe.moph.go.th/boedb/surdata/disease.php?dcontent=old&ds=262766.

49. Halstead, S.B., Mahalingam, S., Marovich, M.A., Ubol, S., and Mosser, D.M., *Intrinsic antibody-dependent enhancement of microbial infection in macrophages: disease regulation by immune complexes.* Lancet Infect Dis, 2010. **10**(10): p. 712-22.

50. Halstead, S.B., Heinz, F.X., Barrett, A.D., and Roehrig, J.T., *Dengue virus: molecular basis of cell entry and pathogenesis, 25-27 June 2003, Vienna, Austria.* Vaccine, 2005. **23**(7): p. 849-56.

51. Tang, K.F. and Ooi, E.E., *Diagnosis of dengue: an update.* Expert Rev Anti Infect Ther, 2012. **10**(8): p. 895-907.

52. Phuong, H.L., de Vries, P.J., Nagelkerke, N., Giao, P.T., Hung le, Q., Binh, T.Q., Nga, T.T., Nam, N.V., and Kager, P.A., *Acute undifferentiated fever in Binh Thuan province, Vietnam: imprecise clinical diagnosis and irrational pharmaco-therapy.* Trop Med Int Health, 2006. **11**(6): p. 869-79.

53. World Health Organization (WHO). *Dengue haemorrhagoc fever: diagnosis, treatment and control (second edition). World Health Organization, geneva, Switzerland.* 1997; Available from: http://www.who.int/csr/resources/publications/dengue/Denguepublication/en/.

54. Bandyopadhyay, S., Lum, L.C., and Kroeger, A., *Classifying dengue: a review of the difficulties in using the WHO case classification for dengue haemorrhagic fever.* Trop Med Int Health, 2006. **11**(8): p. 1238-55.

55. Watt, G., Jongsakul, K., Chouriyagune, C., and Paris, R., *Differentiating dengue virus infection from scrub typhus in Thai adults with fever.* Am J Trop Med Hyg, 2003. **68**(5): p. 536-8.

56. Wichmann, O., Stark, K., Shu, P.Y., Niedrig, M., Frank, C., Huang, J.H., and Jelinek, T., *Clinical features and pitfalls in the laboratory diagnosis of dengue in travellers.* BMC Infect Dis, 2006. **6**: p. 120.

57. Peeling, R.W., Artsob, H., Pelegrino, J.L., Buchy, P., Cardosa, M.J., Devi, S., Enria, D.A., Farrar, J., Gubler, D.J., Guzman, M.G., Halstead, S.B., Hunsperger, E., Kliks, S., Margolis, H.S., Nathanson, C.M., Nguyen, V.C., Rizzo, N., Vazquez, S., and Yoksan, S., *Evaluation of diagnostic tests: dengue.* Nat Rev Microbiol, 2010. **8**(12 Suppl): p. S30-8.

58. De Paula, S.O. and Fonseca, B.A., *Dengue: a review of the laboratory tests a clinician must know to achieve a correct diagnosis.* Braz J Infect Dis, 2004. **8**(6): p. 390-8.

59. Jarman, R.G., Nisalak, A., Anderson, K.B., Klungthong, C., Thaisomboonsuk, B., Kaneechit, W., Kalayanarooj, S., and Gibbons, R.V., *Factors influencing dengue virus isolation by C6/36 cell culture and mosquito inoculation of nested PCR-positive clinical samples.* Am J Trop Med Hyg, 2011. **84**(2): p. 218-23.

60. Amorim, J.H., Alves, R.P., Boscardin, S.B., and Ferreira, L.C., *The dengue virus non-structural 1 protein: risks and benefits.* Virus Res, 2014. **181**: p. 53-60.

61. Alcon, S., Talarmin, A., Debruyne, M., Falconar, A., Deubel, V., and Flamand, M., *Enzyme-linked immunosorbent assay specific to Dengue virus type 1 nonstructural protein NS1 reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencing primary or secondary infections.* J Clin Microbiol, 2002. **40**(2): p. 376-81.

62. Xu, H., Di, B., Pan, Y.X., Qiu, L.W., Wang, Y.D., Hao, W., He, L.J., Yuen, K.Y., and Che, X.Y., *Serotype 1-specific monoclonal antibody-based antigen capture immunoassay for detection of circulating nonstructural protein NS1: Implications for early diagnosis and serotyping of dengue virus infections.* J Clin Microbiol, 2006. **44**(8): p. 2872-8.

63. Libraty, D.H., Young, P.R., Pickering, D., Endy, T.P., Kalayanarooj, S., Green, S., Vaughn, D.W., Nisalak, A., Ennis, F.A., and Rothman, A.L., *High circulating levels of the dengue virus nonstructural protein NS1 early in dengue illness correlate with the development of dengue hemorrhagic fever.* J Infect Dis, 2002. **186**(8): p. 1165-8.

64. Hang, V.T., Nguyet, N.M., Trung, D.T., Tricou, V., Yoksan, S., Dung, N.M., Van Ngoc, T., Hien, T.T., Farrar, J., Wills, B., and Simmons, C.P., *Diagnostic accuracy of NS1 ELISA and lateral flow rapid tests for dengue sensitivity, specificity and relationship to viraemia and antibody responses.* PLoS Negl Trop Dis, 2009. **3**(1): p. e360.

65. Chua, K.B., Mustafa, B., Abdul Wahab, A.H., Chem, Y.K., Khairul, A.H., Kumarasamy, V., Mariam, M., Nurhasmimi, H., and Abdul Rasid, K., *A comparative evaluation of dengue diagnostic tests based on single-acute serum*

samples for laboratory confirmation of acute dengue. Malays J Pathol, 2011. **33**(1): p. 13-20.

66. Osorio, L., Ramirez, M., Bonelo, A., Villar, L.A., and Parra, B., *Comparison of the diagnostic accuracy of commercial NS1-based diagnostic tests for early dengue infection.* Virol J, 2010. **7**: p. 361.

67. Zhang, H., Li, W., Wang, J., Peng, H., Che, X., Chen, X., and Zhou, Y., *NS1-based tests with diagnostic utility for confirming dengue infection: a meta-analysis.* Int J Infect Dis, 2014. **26**: p. 57-66.

68. Saito, Y., Moi, M.L., Kotaki, A., Ikeda, M., Tajima, S., Shiba, H., Hosono, K., Saijo, M., Kurane, I., and Takasaki, T., *Detection of Dengue Virus Nonstructural Protein 1 (NS1) in Urine Samples using ELISA as a Laboratory Diagnostic Method for Dengue Virus Infection.* Jpn J Infect Dis, 2015.

69. Rainwater-Lovett, K., Rodriguez-Barraquer, I., Cummings, D.A., and Lessler, J., *Variation in dengue virus plaque reduction neutralization testing: systematic review and pooled analysis.* BMC Infect Dis, 2012. **12**: p. 233.

70. Innis, B.L., Nisalak, A., Nimmannitya, S., Kusalerdchariya, S., Chongswasdi, V., Suntayakorn, S., Puttisri, P., and Hoke, C.H., *An enzyme-linked immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis co-circulate.* Am J Trop Med Hyg, 1989. **40**(4): p. 418-27.

71. Blacksell, S.D., *Commercial dengue rapid diagnostic tests for point-of-care application: recent evaluations and future needs?* J Biomed Biotechnol, 2012. **2012**: p. 151967.

72. Blacksell, S.D., Jarman, R.G., Gibbons, R.V., Tanganuchitcharnchai, A., Mammen, M.P., Jr., Nisalak, A., Kalayanarooj, S., Bailey, M.S., Premaratna, R., de Silva, H.J., Day, N.P., and Lalloo, D.G., *Comparison of seven commercial antigen and*

antibody enzyme-linked immunosorbent assays for detection of acute dengue infection. Clin Vaccine Immunol, 2012. **19**(5): p. 804-10.

73. Hunsperger, E.A., Yoksan, S., Buchy, P., Nguyen, V.C., Sekaran, S.D., Enria, D.A., Pelegrino, J.L., Vazquez, S., Artsob, H., Drebot, M., Gubler, D.J., Halstead, S.B., Guzman, M.G., Margolis, H.S., Nathanson, C.M., Rizzo Lic, N.R., Bessoff, K.E., Kliks, S., and Peeling, R.W., *Evaluation of commercially available anti-dengue virus immunoglobulin M tests.* Emerg Infect Dis, 2009. **15**(3): p. 436-40.

74. Blacksell, S.D., Newton, P.N., Bell, D., Kelley, J., Mammen, M.P., Jr., Vaughn, D.W., Wuthiekanun, V., Sungkakum, A., Nisalak, A., and Day, N.P., *The comparative accuracy of 8 commercial rapid immunochromatographic assays for the diagnosis of acute dengue virus infection.* Clin Infect Dis, 2006. **42**(8): p. 1127-34.

75. Wu, S.J., Paxton, H., Hanson, B., Kung, C.G., Chen, T.B., Rossi, C., Vaughn, D.W., Murphy, G.S., and Hayes, C.G., *Comparison of two rapid diagnostic assays for detection of immunoglobulin M antibodies to dengue virus.* Clin Diagn Lab Immunol, 2000. **7**(1): p. 106-10.

76. Yap, G., Sil, B.K., and Ng, L.C., *Use of saliva for early dengue diagnosis.* PLoS Negl Trop Dis, 2011. **5**(5): p. e1046.

77. Blacksell, S.D., Jarman, R.G., Bailey, M.S., Tanganuchitcharnchai, A., Jenjaroen, K., Gibbons, R.V., Paris, D.H., Premaratna, R., de Silva, H.J., Lalloo, D.G., and Day, N.P., *Evaluation of six commercial point-of-care tests for diagnosis of acute dengue infections: the need for combining NS1 antigen and IgM/IgG antibody detection to achieve acceptable levels of accuracy.* Clin Vaccine Immunol, 2011. **18**(12): p. 2095-101.

78. Fry, S.R., Meyer, M., Semple, M.G., Simmons, C.P., Sekaran, S.D., Huang, J.X., McElnea, C., Huang, C.Y., Valks, A., Young, P.R., and Cooper, M.A., *The*

diagnostic sensitivity of dengue rapid test assays is significantly enhanced by using a combined antigen and antibody testing approach. PLoS Negl Trop Dis, 2011. **5**(6): p. e1199.

79. Wang, S.M. and Sekaran, S.D., *Early diagnosis of Dengue infection using a commercial Dengue Duo rapid test kit for the detection of NS1, IGM, and IGG.* Am J Trop Med Hyg, 2010. **83**(3): p. 690-5.

80. Lanciotti, R.S., Calisher, C.H., Gubler, D.J., Chang, G.J., and Vorndam, A.V., *Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction.* J Clin Microbiol, 1992. **30**(3): p. 545-51.

81. Harris, E., Roberts, T.G., Smith, L., Selle, J., Kramer, L.D., Valle, S., Sandoval, E., and Balmaseda, A., *Typing of dengue viruses in clinical specimens and mosquitoes by single-tube multiplex reverse transcriptase PCR.* J Clin Microbiol, 1998. **36**(9): p. 2634-9.

82. Laue, T., Emmerich, P., and Schmitz, H., *Detection of dengue virus RNA in patients after primary or secondary dengue infection by using the TaqMan automated amplification system.* J Clin Microbiol, 1999. **37**(8): p. 2543-7.

83. Callahan, J.D., Wu, S.J., Dion-Schultz, A., Mangold, B.E., Peruski, L.F., Watts, D.M., Porter, K.R., Murphy, G.R., Suharyono, W., King, C.C., Hayes, C.G., and Temenak, J.J., *Development and evaluation of serotype- and group-specific fluorogenic reverse transcriptase PCR (TaqMan) assays for dengue virus.* J Clin Microbiol, 2001. **39**(11): p. 4119-24.

84. Drosten, C., Gottig, S., Schilling, S., Asper, M., Panning, M., Schmitz, H., and Gunther, S., *Rapid detection and quantification of RNA of Ebola and Marburg viruses, Lassa virus, Crimean-Congo hemorrhagic fever virus, Rift Valley fever*

virus, dengue virus, and yellow fever virus by real-time reverse transcription-PCR. J Clin Microbiol, 2002. **40**(7): p. 2323-30.

85. Morita, K., Tanaka, M., and Igarashi, A., *Rapid identification of dengue virus serotypes by using polymerase chain reaction.* J Clin Microbiol, 1991. **29**(10): p. 2107-10.

86. Shu, P.Y., Chang, S.F., Kuo, Y.C., Yuen, Y.Y., Chien, L.J., Sue, C.L., Lin, T.H., and Huang, J.H., *Development of Group- and Serotype-Specific One-Step SYBR Green I-Based Real-Time Reverse Transcription-PCR Assay for Dengue Virus* J Clin Microbiol, 2003. **41**(6): p. 9.

87. De Paula, S.O., Pires Neto, R.J., Correa, J.A., Assumpcao, S.R., Costa, M.L., Lima, D.M., and Fonseca, B.A., *The use of reverse transcription-polymerase chain reaction (RT-PCR) for the rapid detection and identification of dengue virus in an endemic region: a validation study.* Trans R Soc Trop Med Hyg, 2002. **96**(3): p. 266-9.

88. Munoz-Jordan, J.L., Collins, C.S., Vergne, E., Santiago, G.A., Petersen, L., Sun, W., and Linnen, J.M., *Highly sensitive detection of dengue virus nucleic acid in samples from clinically ill patients.* J Clin Microbiol, 2009. **47**(4): p. 927-31.

89. Li, S., Fang, M., Zhou, B., Ni, H., Shen, Q., Zhang, H., Han, Y., Yin, J., Chang, W., Xu, G., and Cao, G., *Simultaneous detection and differentiation of dengue virus serotypes 1-4, Japanese encephalitis virus, and West Nile virus by a combined reverse-transcription loop-mediated isothermal amplification assay.* Virol J, 2011. **8**: p. 360.

90. Parida, M., Horioke, K., Ishida, H., Dash, P.K., Saxena, P., Jana, A.M., Islam, M.A., Inoue, S., Hosaka, N., and Morita, K., *Rapid detection and differentiation of dengue virus serotypes by a real-time reverse transcription-loop-mediated isothermal amplification assay.* J Clin Microbiol, 2005. **43**(6): p. 2895-903.

91. Abd El Wahed, A., Patel, P., Faye, O., Thaloengsok, S., Heidenreich, D., Matangkasombut, P., Manopwisedjaroen, K., Sakuntabhai, A., Sall, A.A., Hufert, F.T., and Weidmann, M., *Recombinase Polymerase Amplification Assay for Rapid Diagnostics of Dengue Infection.* PLoS One, 2015. **10**(6): p. e0129682.

92. De Paula, S.O. and Lopes da Fonseca, B.A., *Optimizing dengue diagnosis by RT-PCR in IgM-positive samples: comparison of whole blood, buffy-coat and serum as clinical samples.* J Virol Methods, 2002. **102**(1-2): p. 113-7.

93. Klungthong, C., Gibbons, R.V., Thaisomboonsuk, B., Nisalak, A., Kalayanarooj, S., Thirawuth, V., Nutkumhang, N., Mammen, M.P., Jr., and Jarman, R.G., *Dengue virus detection using whole blood for reverse transcriptase PCR and virus isolation.* J Clin Microbiol, 2007. **45**(8): p. 2480-5.

94. Bharti, A.R., Nally, J.E., Ricaldi, J.N., Matthias, M.A., Diaz, M.M., Lovett, M.A., Levett, P.N., Gilman, R.H., Willig, M.R., Gotuzzo, E., and Vinetz, J.M., *Leptospirosis: a zoonotic disease of global importance.* Lancet Infect Dis, 2003. **3**(12): p. 757-71.

95. Haake, D.A., *Spirochaetal lipoproteins and pathogenesis.* Microbiology, 2000. **146 (Pt 7)**: p. 1491-504.

96. Adler, B. and de la Pena Moctezuma, A., *Leptospira and leptospirosis.* Vet Microbiol, 2010. **140**(3-4): p. 287-96.

97. Cerqueira, G.M. and Picardeau, M., *A century of Leptospira strain typing.* Infect Genet Evol, 2009. **9**(5): p. 760-8.

98. Levett, P.N., *Leptospirosis.* Clin Microbiol Rev, 2001. **14**(2): p. 296-326.

99. Cinco, M., *New insights into the pathogenicity of leptospires: evasion of host defences.* New Microbiol, 2010. **33**(4): p. 283-92.

100. Slack, A.T., Khairani-Bejo, S., Symonds, M.L., Dohnt, M.F., Galloway, R.L., Steigerwalt, A.G., Bahaman, A.R., Craig, S., Harrower, B.J., and Smythe, L.D.,

Leptospira kmetyi sp. nov., isolated from an environmental source in Malaysia. Int J Syst Evol Microbiol, 2009. **59**(Pt 4): p. 705-8.

101. International Leptospirosis Society. *Leptospiral Nomenclature*. [cited 2016 July]; Available from: http://www.leptosociety.org/resources.

102. Ren, S.X., Fu, G., Jiang, X.G., Zeng, R., Miao, Y.G., Xu, H., Zhang, Y.X., Xiong, H., Lu, G., Lu, L.F., Jiang, H.Q., Jia, J., Tu, Y.F., Jiang, J.X., Gu, W.Y., Zhang, Y.Q., Cai, Z., Sheng, H.H., Yin, H.F., Zhang, Y., Zhu, G.F., Wan, M., Huang, H.L., Qian, Z., Wang, S.Y., Ma, W., Yao, Z.J., Shen, Y., Qiang, B.Q., Xia, Q.C., Guo, X.K., Danchin, A., Saint Girons, I., Somerville, R.L., Wen, Y.M., Shi, M.H., Chen, Z., Xu, J.G., and Zhao, G.P., *Unique physiological and pathogenic features of Leptospira interrogans revealed by whole-genome sequencing.* Nature, 2003. **422**(6934): p. 888-93.

103. Nascimento, A.L., Ko, A.I., Martins, E.A., Monteiro-Vitorello, C.B., Ho, P.L., Haake, D.A., Verjovski-Almeida, S., Hartskeerl, R.A., Marques, M.V., Oliveira, M.C., Menck, C.F., Leite, L.C., Carrer, H., Coutinho, L.L., Degrave, W.M., Dellagostin, O.A., El-Dorry, H., Ferro, E.S., Ferro, M.I., Furlan, L.R., Gamberini, M., Giglioti, E.A., Goes-Neto, A., Goldman, G.H., Goldman, M.H., Harakava, R., Jeronimo, S.M., Junqueira-de-Azevedo, I.L., Kimura, E.T., Kuramae, E.E., Lemos, E.G., Lemos, M.V., Marino, C.L., Nunes, L.R., de Oliveira, R.C., Pereira, G.G., Reis, M.S., Schriefer, A., Siqueira, W.J., Sommer, P., Tsai, S.M., Simpson, A.J., Ferro, J.A., Camargo, L.E., Kitajima, J.P., Setubal, J.C., and Van Sluys, M.A., *Comparative genomics of two Leptospira interrogans serovars reveals novel insights into physiology and pathogenesis.* J Bacteriol, 2004. **186**(7): p. 2164-72.

104. Bulach, D.M., Zuerner, R.L., Wilson, P., Seemann, T., McGrath, A., Cullen, P.A., Davis, J., Johnson, M., Kuczek, E., Alt, D.P., Peterson-Burch, B., Coppel, R.L., Rood, J.I., Davies, J.K., and Adler, B., *Genome reduction in Leptospira*

borgpetersenii reflects limited transmission potential. Proc Natl Acad Sci U S A, 2006. **103**(39): p. 14560-5.

105. Picardeau, M., Bulach, D.M., Bouchier, C., Zuerner, R.L., Zidane, N., Wilson, P.J., Creno, S., Kuczek, E.S., Bommezzadri, S., Davis, J.C., McGrath, A., Johnson, M.J., Boursaux-Eude, C., Seemann, T., Rouy, Z., Coppel, R.L., Rood, J.I., Lajus, A., Davies, J.K., Medigue, C., and Adler, B., *Genome sequence of the saprophyte Leptospira biflexa provides insights into the evolution of Leptospira and the pathogenesis of leptospirosis.* PLoS One, 2008. **3**(2): p. e1607.

106. Bandara, M., Ananda, M., Wickramage, K., Berger, E., and Agampodi, S., *Globalization of leptospirosis through travel and migration.* Global Health, 2014. **10**: p. 61.

107. Haake, D.A. and Levett, P.N., *Leptospirosis in humans.* Curr Top Microbiol Immunol, 2015. **387**: p. 65-97.

108. World Health Organization. *Human leptospirosis: guidance for diagnosis, surveillance and control.* 2003 [cited 2011 31 October]; Available from: http://whqlibdoc.who.int/hq/2003/WHO_CDS_CSR_EPH_2002.23.pdf.

109. Mendoza, M.T., Roxas, E.A., Ginete, J.K., Alejandria, M.M., Roman, A.D., Leyritana, K.T., Penamora, M.A., and Pineda, C.C., *Clinical profile of patients diagnosed with leptospirosis after a typhoon: a multicenter study.* Southeast Asian J Trop Med Public Health, 2013. **44**(6): p. 1021-35.

110. Picardeau, M., Bertherat, E., Jancloes, M., Skouloudis, A.N., Durski, K., and Hartskeerl, R.A., *Rapid tests for diagnosis of leptospirosis: current tools and emerging technologies.* Diagn Microbiol Infect Dis, 2014. **78**(1): p. 1-8.

111. World Health Organization (WHO), *Report of the second meeting of leptospirosis burden epidemiology reference group*. 2011: Geneva, Switzerland.

112. Pappas, G., Papadimitriou, P., Siozopoulou, V., Christou, L., and Akritidis, N., *The globalization of leptospirosis: worldwide incidence trends.* Int J Infect Dis, 2008. **12**(4): p. 351-7.

113. Centre of Epidemiological Information, Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand. *National Disease Surveillance (Report 506)*. [cited 20 Dec 2015; Available from: http://www.boe.moph.go.th/boedb/surdata/.

114. Suwanpakdee, S., Kaewkungwal, J., White, L.J., Asensio, N., Ratanakorn, P., Singhasivanon, P., Day, N.P., and Pan-Ngum, W., *Spatio-temporal patterns of leptospirosis in Thailand: is flooding a risk factor?* Epidemiol Infect, 2015. **143**(10): p. 2106-15.

115. Wuthiekanun, V., Sirisukkarn, N., Daengsupa, P., Sakaraserane, P., Sangkakam, A., Chierakul, W., Smythe, L.D., Symonds, M.L., Dohnt, M.F., Slack, A.T., Day, N.P., and Peacock, S.J., *Clinical diagnosis and geographic distribution of leptospirosis, Thailand.* Emerg Infect Dis, 2007. **13**(1): p. 124-6.

116. Turner, L.H., *Leptospirosis. I.* Trans R Soc Trop Med Hyg, 1967. **61**(6): p. 842-55.

117. Bovet, P., Yersin, C., Merien, F., Davis, C.E., and Perolat, P., *Factors associated with clinical leptospirosis: a population-based case-control study in the Seychelles (Indian Ocean).* Int J Epidemiol, 1999. **28**(3): p. 583-90.

118. Sharma, K.K. and Kalawat, U., *Early diagnosis of leptospirosis by conventional methods: one-year prospective study.* Indian J Pathol Microbiol, 2008. **51**(2): p. 209-11.

119. Ahmad, S.N., Shah, S., and Ahmad, F.M., *Laboratory diagnosis of leptospirosis.* J Postgrad Med, 2005. **51**(3): p. 195-200.

120. Wuthiekanun, V., Chierakul, W., Limmathurotsakul, D., Smythe, L.D., Symonds, M.L., Dohnt, M.F., Slack, A.T., Limpaiboon, R., Suputtamongkol, Y., White, N.J.,

Day, N.P., and Peacock, S.J., *Optimization of culture of Leptospira from humans with leptospirosis.* J Clin Microbiol, 2007. **45**(4): p. 1363-5.

121. Wuthiekanun, V., Amornchai, P., Paris, D.H., Langla, S., Thaipadunpanit, J., Chierakul, W., Smythe, L.D., White, N.J., Day, N.P., Limmathurotsakul, D., and Peacock, S.J., *Rapid isolation and susceptibility testing of Leptospira spp. using a new solid medium, LVW agar.* Antimicrob Agents Chemother, 2013. **57**(1): p. 297-302.

122. Brown, P.D. and Levett, P.N., *Differentiation of Leptospira species and serovars by PCR-restriction endonuclease analysis, arbitrarily primed PCR and low-stringency PCR.* J Med Microbiol, 1997. **46**(2): p. 173-81.

123. Djadid, N.D., Ganji, Z.F., Gouya, M.M., Rezvani, M., and Zakeri, S., *A simple and rapid nested polymerase chain reaction-restriction fragment length polymorphism technique for differentiation of pathogenic and nonpathogenic Leptospira spp.* Diagn Microbiol Infect Dis, 2009. **63**(3): p. 251-6.

124. Turk, N., Milas, Z., Mojcec, V., Ruzic-Sabljic, E., Staresina, V., Stritof, Z., Habus, J., and Postic, D., *Molecular analysis of Leptospira spp. isolated from humans by restriction fragment length polymorphism, real-time PCR and pulsed-field gel electrophoresis.* FEMS Microbiol Lett, 2009. **300**(2): p. 174-9.

125. Sonthayanon, P., Chierakul, W., Wuthiekanun, V., Thaipadungpanit, J., Kalambaheti, T., Boonsilp, S., Amornchai, P., Smythe, L.D., Limmathurotsakul, D., Day, N.P., and Peacock, S.J., *Accuracy of loop-mediated isothermal amplification for diagnosis of human leptospirosis in Thailand.* Am J Trop Med Hyg, 2011. **84**(4): p. 614-20.

126. Wangroongsarb, P., Chanket, T., Gunlabun, K., Long do, H., Satheanmethakul, P., Jetanadee, S., Thaipadungpanit, J., Wuthiekanun, V., Peacock, S.J., Blacksell, S.D., Smythe, L.D., Bulach, D.M., and Kalambaheti, T., *Molecular typing of Leptospira*

spp. based on putative O-antigen polymerase gene (wzy), the benefit over 16S rRNA gene sequence. FEMS Microbiol Lett, 2007. **271**(2): p. 170-9.

127. Ahmed, N., Devi, S.M., Valverde Mde, L., Vijayachari, P., Machang'u, R.S., Ellis, W.A., and Hartskeerl, R.A., *Multilocus sequence typing method for identification and genotypic classification of pathogenic Leptospira species.* Ann Clin Microbiol Antimicrob, 2006. **5**: p. 28.

128. Thaipadungpanit, J., Wuthiekanun, V., Chierakul, W., Smythe, L.D., Petkanchanapong, W., Limpaiboon, R., Apiwatanaporn, A., Slack, A.T., Suputtamongkol, Y., White, N.J., Feil, E.J., Day, N.P., and Peacock, S.J., *A dominant clone of Leptospira interrogans associated with an outbreak of human leptospirosis in Thailand.* PLoS Negl Trop Dis, 2007. **1**(1): p. e56.

129. Cumberland, P., Everard, C.O., and Levett, P.N., *Assessment of the efficacy of an IgM-elisa and microscopic agglutination test (MAT) in the diagnosis of acute leptospirosis.* Am J Trop Med Hyg, 1999. **61**(5): p. 731-4.

130. Doungchawee, G., Kositanont, U., Niwetpathomwat, A., Inwisai, T., Sagarasaeranee, P., and Haake, D.A., *Early diagnosis of leptospirosis by immunoglobulin M immunoblot testing.* Clin Vaccine Immunol, 2008. **15**(3): p. 492-8.

131. Bajani, M.D., Ashford, D.A., Bragg, S.L., Woods, C.W., Aye, T., Spiegel, R.A., Plikaytis, B.D., Perkins, B.A., Phelan, M., Levett, P.N., and Weyant, R.S., *Evaluation of four commercially available rapid serologic tests for diagnosis of leptospirosis.* J Clin Microbiol, 2003. **41**(2): p. 803-9.

132. Murray, C.K., Gray, M.R., Mende, K., Parker, T.M., Samir, A., Rahman, B.A., Habashy, E.E., Hospenthal, D.R., and Pimentel, G., *Use of patient-specific Leptospira isolates in the diagnosis of leptospirosis employing microscopic agglutination testing (MAT).* Trans R Soc Trop Med Hyg, 2011. **105**(4): p. 209-13.

133. Smythe, L.D., Wuthiekanun, V., Chierakul, W., Suputtamongkol, Y., Tiengrim, S., Dohnt, M.F., Symonds, M.L., Slack, A.T., Apiwattanaporn, A., Chueasuwanchai, S., Day, N.P., and Peacock, S.J., *The microscopic agglutination test (MAT) is an unreliable predictor of infecting Leptospira serovar in Thailand.* Am J Trop Med Hyg, 2009. **81**(4): p. 695-7.

134. Winslow, W.E., Merry, D.J., Pirc, M.L., and Devine, P.L., *Evaluation of a commercial enzyme-linked immunosorbent assay for detection of immunoglobulin M antibody in diagnosis of human leptospiral infection.* J Clin Microbiol, 1997. **35**(8): p. 1938-42.

135. Gussenhoven, G.C., van der Hoorn, M.A., Goris, M.G., Terpstra, W.J., Hartskeerl, R.A., Mol, B.W., van Ingen, C.W., and Smits, H.L., *LEPTO dipstick, a dipstick assay for detection of Leptospira-specific immunoglobulin M antibodies in human sera.* J Clin Microbiol, 1997. **35**(1): p. 92-7.

136. Effler, P.V., Bogard, A.K., Domen, H.Y., Katz, A.R., Higa, H.Y., and Sasaki, D.M., *Evaluation of eight rapid screening tests for acute leptospirosis in Hawaii.* J Clin Microbiol, 2002. **40**(4): p. 1464-9.

137. Levett, P.N. and Whittington, C.U., *Evaluation of the indirect hemagglutination assay for diagnosis of acute leptospirosis.* J Clin Microbiol, 1998. **36**(1): p. 11-4.

138. Senthilkumar, T., Subathra, M., Phil, M., Ramadass, P., and Ramaswamy, V., *Rapid serodiagnosis of leptospirosis by latex agglutination test and flow-through assay.* Indian J Med Microbiol, 2008. **26**(1): p. 45-9.

139. Sehgal, S.C., Vijayachari, P., Sugunan, A.P., and Umapathi, T., *Field application of Lepto lateral flow for rapid diagnosis of leptospirosis.* J Med Microbiol, 2003. **52**(Pt 10): p. 897-901.

140. Blacksell, S.D., Smythe, L., Phetsouvanh, R., Dohnt, M., Hartskeerl, R., Symonds, M., Slack, A., Vongsouvath, M., Davong, V., Lattana, O., Phongmany, S.,

Keolouangkot, V., White, N.J., Day, N.P., and Newton, P.N., *Limited diagnostic capacities of two commercial assays for the detection of Leptospira immunoglobulin M antibodies in Laos.* Clin Vaccine Immunol, 2006. **13**(10): p. 1166-9.

141. Appassakij, H., Silpapojakul, K., Wansit, R., and Woodtayakorn, J., *Evaluation of the immunofluorescent antibody test for the diagnosis of human leptospirosis.* Am J Trop Med Hyg, 1995. **52**(4): p. 340-3.

142. Kawaoka, Y., Naiki, M., and Yanagawa, R., *Radioimmunoassay system using a serovar-specific lipopolysaccharide antigen of Leptospira.* J Clin Microbiol, 1979. **10**(3): p. 313-6.

143. Myers, D.M., *Serodiagnosis of human leptospirosis by counterimmunoelectrophoresis.* J Clin Microbiol, 1987. **25**(5): p. 897-9.

144. Obregon Fuentes, A.M., Lopez Acosta, C., and Perez Herrera, C., *[Evaluation of the counterimmunoelectrophoresis technique for the serologic diagnosis of leptospirosis].* Rev Cubana Med Trop, 1993. **45**(1): p. 67-71.

145. Banfi, E., Cinco, M., Delia, S., Castagnari, L., Vullo, V., Mastroianni, C.M., and Contini, C., *New trends in the rapid serodiagnosis of leptospirosis.* Zentralbl Bakteriol Mikrobiol Hyg A, 1984. **257**(4): p. 503-7.

146. Goris, M.G., Leeflang, M.M., Loden, M., Wagenaar, J.F., Klatser, P.R., Hartskeerl, R.A., and Boer, K.R., *Prospective evaluation of three rapid diagnostic tests for diagnosis of human leptospirosis.* PLoS Negl Trop Dis, 2013. **7**(7): p. e2290.

147. Smythe, L.D., Smith, I.L., Smith, G.A., Dohnt, M.F., Symonds, M.L., Barnett, L.J., and McKay, D.B., *A quantitative PCR (TaqMan) assay for pathogenic Leptospira spp.* BMC Infect Dis, 2002. **2**: p. 13.

148. Stoddard, R.A., Gee, J.E., Wilkins, P.P., McCaustland, K., and Hoffmaster, A.R., *Detection of pathogenic Leptospira spp. through TaqMan polymerase chain*

reaction targeting the LipL32 gene. Diagn Microbiol Infect Dis, 2009. **64**(3): p. 247-55.

149. Thaipadungpanit, J., Chierakul, W., Wuthiekanun, V., Limmathurotsakul, D., Amornchai, P., Boonslip, S., Smythe, L.D., Limpaiboon, R., Hoffmaster, A.R., Day, N.P., and Peacock, S.J., *Diagnostic accuracy of real-time PCR assays targeting 16S rRNA and lipL32 genes for human leptospirosis in Thailand: a case-control study.* PLoS One, 2011. **6**(1): p. e16236.

150. Slack, A., Symonds, M., Dohnt, M., Harris, C., Brookes, D., and Smythe, L., *Evaluation of a modified Taqman assay detecting pathogenic Leptospira spp. against culture and Leptospira-specific IgM enzyme-linked immunosorbent assay in a clinical environment.* Diagn Microbiol Infect Dis, 2007. **57**(4): p. 361-6.

151. Lin, X., Chen, Y., Lu, Y., and Yan, J., *Application of a loop-mediated isothermal amplification method for the detection of pathogenic Leptospira.* Diagn Microbiol Infect Dis, 2009. **63**(3): p. 237-42.

152. Luce-Fedrow, A., Mullins, K., Kostik, A.P., St John, H.K., Jiang, J., and Richards, A.L., *Strategies for detecting rickettsiae and diagnosing rickettsial diseases.* Future Microbiol, 2015. **10**(4): p. 537-64.

153. Gimenez, D.F., *Staining Rickettsiae in Yolk-Sac Cultures.* Stain Technol, 1964. **39**: p. 135-40.

154. Raoult, D. and Roux, V., *Rickettsioses as paradigms of new or emerging infectious diseases.* Clin Microbiol Rev, 1997. **10**(4): p. 694-719.

155. Brenner, D.J., O'Connor, S.P., Winkler, H.H., and Steigerwalt, A.G., *Proposals to unify the genera Bartonella and Rochalimaea, with descriptions of Bartonella quintana comb. nov., Bartonella vinsonii comb. nov., Bartonella henselae comb. nov., and Bartonella elizabethae comb. nov., and to remove the family*

Bartonellaceae from the order Rickettsiales. Int J Syst Bacteriol, 1993. **43**(4): p. 777-86.

156. Parola, P., Paddock, C.D., and Raoult, D., *Tick-borne rickettsioses around the world: emerging diseases challenging old concepts.* Clin Microbiol Rev, 2005. **18**(4): p. 719-56.

157. Fournier, P.E. and Raoult, D., *Current knowledge on phylogeny and taxonomy of Rickettsia spp.* Ann N Y Acad Sci, 2009. **1166**: p. 1-11.

158. Parola, P., Davoust, B., and Raoult, D., *Tick- and flea-borne rickettsial emerging zoonoses.* Vet Res, 2005. **36**(3): p. 469-92.

159. Aurélie, R., Oleg, M., and Didier, R., *Old and new tick-borne rickettsioses.* Trans R Soc Trop Med Hyg, 2009. **1**(1): p. 17-25.

160. Merhej, V., Angelakis, E., Socolovschi, C., and Raoult, D., *Genotyping, evolution and epidemiological findings of Rickettsia species.* Infect Genet Evol, 2014. **25**: p. 122-37.

161. Tamura, A., Ohashi, N., Urakami, H., and Miyamura, S., *Classification of Rickettsia tsutsugamushi in a new genus, Orientia gen. nov., as Orientia tsutsugamushi comb. nov.* Int J Syst Bacteriol, 1995. **45**(3): p. 589-91.

162. Izzard, L., Fuller, A., Blacksell, S.D., Paris, D.H., Richards, A.L., Aukkanit, N., Nguyen, C., Jiang, J., Fenwick, S., Day, N.P., Graves, S., and Stenos, J., *Isolation of a novel Orientia species (O. chuto sp. nov.) from a patient infected in Dubai.* J Clin Microbiol, 2010. **48**(12): p. 4404-9.

163. Balcells, M.E., Rabagliati, R., Garcia, P., Poggi, H., Oddo, D., Concha, M., Abarca, K., Jiang, J., Kelly, D.J., Richards, A.L., and Fuerst, P.A., *Endemic scrub typhus-like illness, Chile.* Emerg Infect Dis, 2011. **17**(9): p. 1659-63.

164. Darby, A.C., Cho, N.H., Fuxelius, H.H., Westberg, J., and Andersson, S.G., *Intracellular pathogens go extreme: genome evolution in the Rickettsiales.* Trends Genet, 2007. **23**(10): p. 511-20.

165. Andersson, S.G., Zomorodipour, A., Andersson, J.O., Sicheritz-Ponten, T., Alsmark, U.C., Podowski, R.M., Naslund, A.K., Eriksson, A.S., Winkler, H.H., and Kurland, C.G., *The genome sequence of Rickettsia prowazekii and the origin of mitochondria.* Nature, 1998. **396**(6707): p. 133-40.

166. Cho, N.H., Kim, H.R., Lee, J.H., Kim, S.Y., Kim, J., Cha, S., Kim, S.Y., Darby, A.C., Fuxelius, H.H., Yin, J., Kim, J.H., Kim, J., Lee, S.J., Koh, Y.S., Jang, W.J., Park, K.H., Andersson, S.G., Choi, M.S., and Kim, I.S., *The Orientia tsutsugamushi genome reveals massive proliferation of conjugative type IV secretion system and host-cell interaction genes.* Proc Natl Acad Sci U S A, 2007. **104**(19): p. 7981-6.

167. Watt, G. and Parola, P., *Scrub typhus and tropical rickettsioses.* Curr Opin Infect Dis, 2003. **16**(5): p. 429-36.

168. Civen, R. and Ngo, V., *Murine typhus: an unrecognized suburban vectorborne disease.* Clin Infect Dis, 2008. **46**(6): p. 913-8.

169. Tamura, A., Urakami, H., and Ohashi, N., *A comparative view of Rickettsia tsutsugamushi and the other groups of rickettsiae.* Eur J Epidemiol, 1991. **7**(3): p. 259-69.

170. Suputtamongkol, Y., Suttinont, C., Niwatayakul, K., Hoontrakul, S., Limpaiboon, R., Chierakul, W., Losuwanaluk, K., and Saisongkork, W., *Epidemiology and clinical aspects of rickettsioses in Thailand.* Ann N Y Acad Sci, 2009. **1166**: p. 172-9.

171. Parola, P., Miller, R.S., McDaniel, P., Telford, S.R., 3rd, Rolain, J.M., Wongsrichanalai, C., and Raoult, D., *Emerging rickettsioses of the Thai-Myanmar border.* Emerg Infect Dis, 2003. **9**(5): p. 592-5.

172. Phongmany, S., Rolain, J.M., Phetsouvanh, R., Blacksell, S.D., Soukkhaseum, V., Rasachack, B., Phiasakha, K., Soukkhaseum, S., Frichithavong, K., Chu, V., Keolouangkhot, V., Martinez-Aussel, B., Chang, K., Darasavath, C., Rattanavong, O., Sisouphone, S., Mayxay, M., Vidamaly, S., Parola, P., Thammavong, C., Heuangvongsy, M., Syhavong, B., Raoult, D., White, N.J., and Newton, P.N., *Rickettsial infections and fever, Vientiane, Laos.* Emerg Infect Dis, 2006. **12**(2): p. 256-62.

173. Silpapojakul, K., *Scrub typhus in the Western Pacific region.* Ann Acad Med Singapore, 1997. **26**(6): p. 794-800.

174. Rosenberg, R., *Drug-resistant scrub typhus: Paradigm and paradox.* Parasitol Today, 1997. **13**(4): p. 131-2.

175. Jiang, J., Sangkasuwan, V., Lerdthusnee, K., Sukwit, S., Chuenchitra, T., Rozmajzl, P.J., Eamsila, C., Jones, J.W., and Richards, A.L., *Human Infection with Rickettsia honei, Thailand.* Emerg Infect Dis, 2005. **11**(9): p. 1473-5.

176. Edouard, S., Bhengsri, S., Dowell, S.F., Watt, G., Parola, P., and Raoult, D., *Two human cases of Rickettsia felis infection, Thailand.* Emerg Infect Dis. **20**(10): p. 1780-1.

177. Choi, Y.J., Jang, W.J., Ryu, J.S., Lee, S.H., Park, K.H., Paik, H.S., Koh, Y.S., Choi, M.S., and Kim, I.S., *Spotted fever group and typhus group rickettsioses in humans, South Korea.* Emerg Infect Dis, 2005. **11**(2): p. 237-44.

178. Angelakis, E., Munasinghe, A., Yaddehige, I., Liyanapathirana, V., Thevanesam, V., Bregliano, A., Socolovschi, C., Edouard, S., Fournier, P.E., Raoult, D., and

Parola, P., *Detection of rickettsioses and Q fever in Sri Lanka.* Am J Trop Med Hyg. **86**(4): p. 711-2.

179. Tsai, K.H., Lu, H.Y., Tsai, J.J., Yu, S.K., Huang, J.H., and Shu, P.Y., *Human case of Rickettsia felis infection, Taiwan.* Emerg Infect Dis, 2008. **14**(12): p. 1970-2.

180. Ben-Zvi, I., Meltzer, E., Nogueras, M.M., Segura, F., and Bank, I., *First detection of human infection with Rickettsia felis in Israel.* Am J Med Sci. **340**(4): p. 343.

181. Seong, S.Y., Choi, M.S., and Kim, I.S., *Orientia tsutsugamushi infection: overview and immune responses.* Microbes Infect, 2001. **3**(1): p. 11-21.

182. Paris, D.H., Shelite, T.R., Day, N.P., and Walker, D.H., *Unresolved problems related to scrub typhus: a seriously neglected life-threatening disease.* Am J Trop Med Hyg, 2013. **89**(2): p. 301-7.

183. Kaushik, R.M., Kaushik, R., and Bhargava, A., *Multiple eschars in scrub typhus.* Trop Med Health, 2014. **42**(2): p. 65-6.

184. Kim, D.M., Won, K.J., Park, C.Y., Yu, K.D., Kim, H.S., Yang, T.Y., Lee, J.H., Kim, H.K., Song, H.J., Lee, S.H., and Shin, H., *Distribution of eschars on the body of scrub typhus patients: a prospective study.* Am J Trop Med Hyg, 2007. **76**(5): p. 806-9.

185. Ogawa, M., Hagiwara, T., Kishimoto, T., Shiga, S., Yoshida, Y., Furuya, Y., Kaiho, I., Ito, T., Nemoto, H., Yamamoto, N., and Masukawa, K., *Scrub typhus in Japan: epidemiology and clinical features of cases reported in 1998.* Am J Trop Med Hyg, 2002. **67**(2): p. 162-5.

186. Silpapojakul, K. and Varachit, B., *Paediatric scrub typhus in Thailand: a study of 73 confirmed cases.* Trans R Soc Trop Med Hyg, 2004. **98**(6): p. 354-9.

187. Sakamoto, N., Nakamura-Uchiyama, F., Kobayashi, K., Takasaki, T., Ogasawara, Y., Ando, S., Iwabuchi, S., and Ohnishi, K., *Severe murine typhus with shock and*

acute respiratory failure in a Japanese traveler after returning from Thailand. J Travel Med, 2013. **20**(1): p. 50-3.

188. Hamaguchi, S., Cuong, N.C., Tra, D.T., Doan, Y.H., Shimizu, K., Tuan, N.Q., Yoshida, L.M., Mai le, Q., Duc-Anh, D., Ando, S., Arikawa, J., Parry, C.M., Ariyoshi, K., and Thuy, P.T., *Clinical and Epidemiological Characteristics of Scrub Typhus and Murine Typhus among Hospitalized Patients with Acute Undifferentiated Fever in Northern Vietnam.* Am J Trop Med Hyg, 2015. **92**(5): p. 972-8.

189. Richards, A.L., *Worldwide detection and identification of new and old rickettsiae and rickettsial diseases.* FEMS Immunol Med Microbiol, 2012. **64**(1): p. 107-10.

190. Bourgeois, A.L., Olson, J.G., Fang, R.C., Huang, J., Wang, C.L., Chow, L., Bechthold, D., Dennis, D.T., Coolbaugh, J.C., and Weiss, E., *Humoral and cellular responses in scrub typhus patients reflecting primary infection and reinfection with Rickettsia tsutsugamushi.* Am J Trop Med Hyg, 1982. **31**(3 Pt 1): p. 532-40.

191. Blacksell, S.D., Bryant, N.J., Paris, D.H., Doust, J.A., Sakoda, Y., and Day, N.P., *Scrub typhus serologic testing with the indirect immunofluorescence method as a diagnostic gold standard: a lack of consensus leads to a lot of confusion.* Clin Infect Dis, 2007. **44**(3): p. 391-401.

192. Lim, C., Blacksell, S.D., Laongnualpanich, A., Kantipong, P., Day, N.P., Paris, D.H., and Limmathurotsakul, D., *Optimal Cutoff Titers for Indirect Immunofluorescence Assay for Diagnosis of Scrub Typhus.* J Clin Microbiol, 2015. **53**(11): p. 3663-6.

193. Vestris, G., Rolain, J.M., Fournier, P.E., Birg, M.L., Enea, M., Patrice, J.Y., and Raoult, D., *Seven years' experience of isolation of Rickettsia spp. from clinical specimens using the shell vial cell culture assay.* Ann N Y Acad Sci, 2003. **990**: p. 371-4.

194. Luksameetanasan, R., Blacksell, S.D., Kalambaheti, T., Wuthiekanun, V., Chierakul, W., Chueasuwanchai, S., Apiwattanaporn, A., Stenos, J., Graves, S., Peacock, S.J., and Day, N.P., *Patient and sample-related factors that effect the success of in vitro isolation of Orientia tsutsugamushi.* Southeast Asian J Trop Med Public Health, 2007. **38**(1): p. 91-6.

195. Lim, C., Paris, D.H., Blacksell, S.D., Laongnualpanich, A., Kantipong, P., Chierakul, W., Wuthiekanun, V., Day, N.P., Cooper, B.S., and Limmathurotsakul, D., *How to Determine the Accuracy of an Alternative Diagnostic Test when It Is Actually Better than the Reference Tests: A Re-Evaluation of Diagnostic Tests for Scrub Typhus Using Bayesian LCMs.* PLoS One, 2015. **10**(5): p. e0114930.

196. Koh, G.C., Maude, R.J., Paris, D.H., Newton, P.N., and Blacksell, S.D., *Diagnosis of scrub typhus.* Am J Trop Med Hyg, 2010. **82**(3): p. 368-70.

197. Amano, K., Suzuki, N., Fujita, M., Nakamura, Y., and Suto, T., *Serological reactivity of sera from scrub typhus patients against Weil-Felix test antigens.* Microbiol Immunol, 1993. **37**(12): p. 927-33.

198. Pradutkanchana, J., Silpapojakul, K., Paxton, H., Pradutkanchana, S., Kelly, D.J., and Strickman, D., *Comparative evaluation of four serodiagnostic tests for scrub typhus in Thailand.* Trans R Soc Trop Med Hyg, 1997. **91**(4): p. 425-8.

199. Brown, G.W., Robinson, D.M., Huxsoll, D.L., Ng, T.S., and Lim, K.J., *Scrub typhus: a common cause of illness in indigenous populations.* Trans R Soc Trop Med Hyg, 1976. **70**(5-6): p. 444-8.

200. Silpapojakul, K., Pradutkanchana, J., Pradutkanchana, S., and Kelly, D.J., *Rapid, simple serodiagnosis of murine typhus.* Trans R Soc Trop Med Hyg, 1995. **89**(6): p. 625-8.

201. Kularatne, S.A. and Gawarammana, I.B., *Validity of the Weil-Felix test in the diagnosis of acute rickettsial infections in Sri Lanka.* Trans R Soc Trop Med Hyg, 2009. **103**(4): p. 423-4.

202. Phetsouvanh, R., Thojaikong, T., Phoumin, P., Sibounheuang, B., Phommasone, K., Chansamouth, V., Lee, S.J., Newton, P.N., and Blacksell, S.D., *Inter- and intra-operator variability in the reading of indirect immunofluorescence assays for the serological diagnosis of scrub typhus and murine typhus.* Am J Trop Med Hyg, 2013. **88**(5): p. 932-6.

203. Radulovic, S., Speed, R., Feng, H.M., Taylor, C., and Walker, D.H., *EIA with species-specific monoclonal antibodies: a novel seroepidemiologic tool for determination of the etiologic agent of spotted fever rickettsiosis.* J Infect Dis, 1993. **168**(5): p. 1292-5.

204. Jang, W.J., Huh, M.S., Park, K.H., Choi, M.S., and Kim, I.S., *Evaluation of an immunoglobulin M capture enzyme-linked immunosorbent assay for diagnosis of Orientia tsutsugamushi infection.* Clin Diagn Lab Immunol, 2003. **10**(3): p. 394-8.

205. Land, M.V., Ching, W.M., Dasch, G.A., Zhang, Z., Kelly, D.J., Graves, S.R., and Devine, P.L., *Evaluation of a commercially available recombinant-protein enzyme-linked immunosorbent assay for detection of antibodies produced in scrub typhus rickettsial infections.* J Clin Microbiol, 2000. **38**(7): p. 2701-5.

206. Coleman, R.E., Sangkasuwan, V., Suwanabun, N., Eamsila, C., Mungviriya, S., Devine, P., Richards, A.L., Rowland, D., Ching, W.M., Sattabongkot, J., and Lerdthusnee, K., *Comparative evaluation of selected diagnostic assays for the detection of IgG and IgM antibody to Orientia tsutsugamushi in Thailand.* Am J Trop Med Hyg, 2002. **67**(5): p. 497-503.

207. Blacksell, S.D., Lim, C., Tanganuchitcharnchai, A., Jintaworn, S., Kantipong, P., Richards, A.L., Paris, D.H., Limmathurotsakul, D., and Day, N.P., *Optimal Cutoff*

and Accuracy of an IgM Enzyme-Linked Immunosorbent Assay for Diagnosis of Acute Scrub Typhus in Northern Thailand: an Alternative Reference Method to the IgM Immunofluorescence Assay. J Clin Microbiol, 2016. **54**(6): p. 1472-8.

208. Blacksell, S.D., Jenjaroen, K., Phetsouvanh, R., Tanganuchitcharnchai, A., Phouminh, P., Phongmany, S., Day, N.P., and Newton, P.N., *Accuracy of rapid IgM-based immunochromatographic and immunoblot assays for diagnosis of acute scrub typhus and murine typhus infections in Laos.* Am J Trop Med Hyg, 2010. **83**(2): p. 365-9.

209. Blacksell, S.D., Jenjaroen, K., Phetsouvanh, R., Wuthiekanun, V., Day, N.P., Newton, P.N., and Ching, W.M., *Accuracy of AccessBio Immunoglobulin M and Total Antibody Rapid Immunochromatographic Assays for the Diagnosis of Acute Scrub Typhus Infection.* Clin Vaccine Immunol, 2009. **17**(2): p. 263-6.

210. Jiang, J., Chan, T.C., Temenak, J.J., Dasch, G.A., Ching, W.M., and Richards, A.L., *Development of a quantitative real-time polymerase chain reaction assay specific for Orientia tsutsugamushi.* Am J Trop Med Hyg, 2004. **70**(4): p. 351-6.

211. Kim, D.M. and Byun, J.N., *Effects of antibiotic treatment on the results of nested PCRs for scrub typhus.* J Clin Microbiol, 2008. **46**(10): p. 3465-6.

212. Kim, D.M., Kim, H.L., Park, C.Y., Yang, T.Y., Lee, J.H., Yang, J.T., Shim, S.K., and Lee, S.H., *Clinical usefulness of eschar polymerase chain reaction for the diagnosis of scrub typhus: a prospective study.* Clin Infect Dis, 2006. **43**(10): p. 1296-300.

213. Lee, S.H., Kim, D.M., Cho, Y.S., Yoon, S.H., and Shim, S.K., *Usefulness of eschar PCR for diagnosis of scrub typhus.* J Clin Microbiol, 2006. **44**(3): p. 1169-71.

214. Furuya, Y., Yoshida, Y., Katayama, T., Yamamoto, S., and Kawamura, A., Jr., *Serotype-specific amplification of Rickettsia tsutsugamushi DNA by nested polymerase chain reaction.* J Clin Microbiol, 1993. **31**(6): p. 1637-40.

215. Kim, D.M., Yun, N.R., Yang, T.Y., Lee, J.H., Yang, J.T., Shim, S.K., Choi, E.N., Park, M.Y., and Lee, S.H., *Usefulness of nested PCR for the diagnosis of scrub typhus in clinical practice: A prospective study.* Am J Trop Med Hyg, 2006. **75**(3): p. 542-5.

216. Sonthayanon, P., Chierakul, W., Wuthiekanun, V., Blacksell, S.D., Pimda, K., Suputtamongkol, Y., Pukrittayakamee, S., White, N.J., Day, N.P., and Peacock, S.J., *Rapid diagnosis of scrub typhus in rural Thailand using polymerase chain reaction.* Am J Trop Med Hyg, 2006. **75**(6): p. 1099-102.

217. Wright, C.L., Nadolny, R.M., Jiang, J., Richards, A.L., Sonenshine, D.E., Gaff, H.D., and Hynes, W.L., *Rickettsia parkeri in gulf coast ticks, southeastern Virginia, USA.* Emerg Infect Dis, 2011. **17**(5): p. 896-8.

218. Henry, K.M., Jiang, J., Rozmajzl, P.J., Azad, A.F., Macaluso, K.R., and Richards, A.L., *Development of quantitative real-time PCR assays to detect Rickettsia typhi and Rickettsia felis, the causative agents of murine typhus and flea-borne spotted fever.* Mol Cell Probes, 2007. **21**(1): p. 17-23.

219. Paris, D.H., Aukkanit, N., Jenjaroen, K., Blacksell, S.D., and Day, N.P., *A highly sensitive quantitative real-time PCR assay based on the groEL gene of contemporary Thai strains of Orientia tsutsugamushi.* Clin Microbiol Infect, 2009. **15**(5): p. 488-95.

220. Paris, D.H., Blacksell, S.D., Stenos, J., Graves, S.R., Unsworth, N.B., Phetsouvanh, R., Newton, P.N., and Day, N.P., *Real-time multiplex PCR assay for detection and differentiation of rickettsiae and orientiae.* Trans R Soc Trop Med Hyg, 2008. **102**(2): p. 186-93.

221. Sonthayanon, P., Chierakul, W., Wuthiekanun, V., Phimda, K., Pukrittayakamee, S., Day, N.P., and Peacock, S.J., *Association of high Orientia tsutsugamushi DNA*

loads with disease of greater severity in adults with scrub typhus. J Clin Microbiol, 2009. **47**(2): p. 430-4.

222. Singhsilarak, T., Leowattana, W., Looareesuwan, S., Wongchotigul, V., Jiang, J., Richards, A.L., and Watt, G., *Short report: detection of Orientia tsutsugamushi in clinical samples by quantitative real-time polymerase chain reaction.* Am J Trop Med Hyg, 2005. **72**(5): p. 640-1.

223. Jiang, J., Stromdahl, E.Y., and Richards, A.L., *Detection of Rickettsia parkeri and Candidatus Rickettsia andeanae in Amblyomma maculatum Gulf Coast ticks collected from humans in the United States.* Vector Borne Zoonotic Dis, 2012. **12**(3): p. 175-82.

224. Prakash, J.A., Sohan Lal, T., Rosemol, V., Verghese, V.P., Pulimood, S.A., Reller, M., and Dumler, J.S., *Molecular detection and analysis of spotted fever group Rickettsia in patients with fever and rash at a tertiary care centre in Tamil Nadu, India.* Pathog Glob Health, 2012. **106**(1): p. 40-5.

225. Tantibhedhyangkul, W., Wongsawat, E., Silpasakorn, S., Waywa, D., Saenyasiri, N., Suesuay, J., Thipmontree, W., and Suputtamongkol, Y., *Use of Multiplex Real-Time PCR To Diagnose Scrub Typhus.* J Clin Microbiol, 2017. **55**(5): p. 1377-1387.

226. Erin, H., Darder, J., Lee, H., Zhiwen, Z., Hua-Wei, C., Wei-Mei, C., and Chien-Chung, C., *Loop-Mediated Isothermal Amplification Assay Targeting the 47-Kda Gene of Orientia tsutsugamushi: A Rapid and Sensitive Alternative to Real-Time PCR.* J Med Microb Diagn, 2012. **1**(4): p. 5.

227. Paris, D.H., Blacksell, S.D., Newton, P.N., and Day, N.P., *Simple, rapid and sensitive detection of Orientia tsutsugamushi by loop-isothermal DNA amplification.* Trans R Soc Trop Med Hyg, 2008. **102**(12): p. 1239-46.

228. Paris, D.H., Blacksell, S.D., Nawtaisong, P., Jenjaroen, K., Teeraratkul, A., Chierakul, W., Wuthiekanun, V., Kantipong, P., and Day, N.P., *Diagnostic Accuracy of a Loop-Mediated Isothermal PCR Assay for Detection of Orientia tsutsugamushi during Acute Scrub Typhus Infection.* PLoS Negl Trop Dis, 2011. **5**(9): p. e1307.

229. Blacksell, S.D., Paris, D.H., Chierakul, W., Wuthiekanun, V., Teeratakul, A., Kantipong, P., and Day, N.P., *Prospective Evaluation of Commercial Antibody-Based Rapid Tests in Combination with a Loop-Mediated Isothermal Amplification PCR Assay for Detection of Orientia tsutsugamushi during the Acute Phase of Scrub Typhus Infection.* Clin Vaccine Immunol, 2012. **19**(3): p. 391-5.

230. Dittrich, S., Castonguay-Vanier, J., Moore, C.E., Thongyoo, N., Newton, P.N., and Paris, D.H., *Loop-mediated isothermal amplification for Rickettsia typhi (the causal agent of murine typhus): problems with diagnosis at the limit of detection.* J Clin Microbiol, 2014. **52**(3): p. 832-8.

231. Chao, C.C., Belinskaya, T., Zhang, Z., and Ching, W.M., *Development of Recombinase Polymerase Amplification Assays for Detection of Orientia tsutsugamushi or Rickettsia typhi.* PLoS Negl Trop Dis, 2015. **9**(7): p. e0003884.

232. Deen, J., von Seidlein, L., Andersen, F., Elle, N., White, N.J., and Lubell, Y., *Community-acquired bacterial bloodstream infections in developing countries in south and southeast Asia: a systematic review.* Lancet Infect Dis, 2012. **12**(6): p. 480-7.

233. Epstein, D., Raveh, D., Schlesinger, Y., Rudensky, B., Gottehrer, N.P., and Yinnon, A.M., *Adult patients with occult bacteremia discharged from the emergency department: epidemiological and clinical characteristics.* Clin Infect Dis, 2001. **32**(4): p. 559-65.

234. Sturmann, K.M., Bopp, J., Molinari, D., Akhtar, S., and Murphy, J., *Blood cultures in adult patients released from an urban emergency department: a 15-month experience.* Acad Emerg Med, 1996. **3**(8): p. 768-75.

235. Onubogu., U.C., Anochie, IC., Akani, NA, *Prevalence of bacteraemia in febrile, under-five children in the children's outpatient clinic of University of Port Harcourt Teaching Hospital, Port Harcourt, Nigeria.* Niger J Paed, 2015. **42**(2): p. 5.

236. Mahende, C., Ngasala, B., Lusingu, J., Butichi, A., Lushino, P., Lemnge, M., Mmbando, B., and Premji, Z., *Bloodstream bacterial infection among outpatient children with acute febrile illness in north-eastern Tanzania.* BMC Res Notes, 2015. **8**: p. 289.

237. Luxemburger, C., Thwai, K.L., White, N.J., Webster, H.K., Kyle, D.E., Maelankirri, L., Chongsuphajaisiddhi, T., and Nosten, F., *The epidemiology of malaria in a Karen population on the western border of Thailand.* Trans R Soc Trop Med Hyg, 1996. **90**(2): p. 105-11.

238. Bousema, T., Okell, L., Felger, I., and Drakeley, C., *Asymptomatic malaria infections: detectability, transmissibility and public health relevance.* Nat Rev Micro, 2014. **12**(12): p. 833-840.

239. Murray, C.K., Gasser, R.A., Jr., Magill, A.J., and Miller, R.S., *Update on rapid diagnostic testing for malaria.* Clin Microbiol Rev, 2008. **21**(1): p. 97-110.

240. Ochola, L.B., Vounatsou, P., Smith, T., Mabaso, M.L., and Newton, C.R., *The reliability of diagnostic techniques in the diagnosis and management of malaria in the absence of a gold standard.* Lancet Infect Dis, 2006. **6**(9): p. 582-8.

241. Kamau, E., Tolbert, L.S., Kortepeter, L., Pratt, M., Nyakoe, N., Muringo, L., Ogutu, B., Waitumbi, J.N., and Ockenhouse, C.F., *Development of a highly sensitive genus-specific quantitative reverse transcriptase real-time PCR assay for*

detection and quantitation of plasmodium by amplifying RNA and DNA of the 18S rRNA genes. J Clin Microbiol, 2011. **49**(8): p. 2946-53.

242. Snounou, G., Viriyakosol, S., Jarra, W., Thaithong, S., and Brown, K.N., *Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections.* Mol Biochem Parasitol, 1993. **58**(2): p. 283-92.

243. Kamau, E., Alemayehu, S., Feghali, K.C., Komisar, J., Regules, J., Cowden, J., and Ockenhouse, C.F., *Measurement of parasitological data by quantitative real-time PCR from controlled human malaria infection trials at the Walter Reed Army Institute of Research.* Malar J, 2014. **13**: p. 288.

244. Imoukhuede, E.B., Andrews, L., Milligan, P., Berthoud, T., Bojang, K., Nwakanma, D., Ismaili, J., Buckee, C., Njie, F., Keita, S., Sowe, M., Lang, T., Gilbert, S.C., Greenwood, B.M., and Hill, A.V., *Low-level malaria infections detected by a sensitive polymerase chain reaction assay and use of this technique in the evaluation of malaria vaccines in an endemic area.* Am J Trop Med Hyg, 2007. **76**(3): p. 486-93.

245. Rougemont, M., Van Saanen, M., Sahli, R., Hinrikson, H.P., Bille, J., and Jaton, K., *Detection of four Plasmodium species in blood from humans by 18S rRNA gene subunit-based and species-specific real-time PCR assays.* J Clin Microbiol, 2004. **42**(12): p. 5636-43.

246. Beshir, K.B., Hallett, R.L., Eziefula, A.C., Bailey, R., Watson, J., Wright, S.G., Chiodini, P.L., Polley, S.D., and Sutherland, C.J., *Measuring the efficacy of anti-malarial drugs in vivo: quantitative PCR measurement of parasite clearance.* Malar J, 2010. **9**: p. 312.

247. Hwang, J., Jaroensuk, J., Leimanis, M.L., Russell, B., McGready, R., Day, N., Snounou, G., Nosten, F., and Imwong, M., *Long-term storage limits PCR-based*

analyses of malaria parasites in archival dried blood spots. Malar J, 2012. **11**: p. 339.

248. Hoyer, S., Nguon, S., Kim, S., Habib, N., Khim, N., Sum, S., Christophel, E.-M., Bjorge, S., Thomson, A., Kheng, S., Chea, N., Yok, S., Top, S., Ros, S., Sophal, U., Thompson, M.M., Mellor, S., Ariey, F., Witkowski, B., Yeang, C., Yeung, S., Duong, S., Newman, R.D., and Menard, D., *Focused Screening and Treatment (FSAT): A PCR-Based Strategy to Detect Malaria Parasite Carriers and Contain Drug Resistant P. falciparum, Pailin, Cambodia.* PLOS ONE, 2012. **7**(10): p. e45797.

249. Imwong, M., Hanchana, S., Malleret, B., Renia, L., Day, N.P., Dondorp, A., Nosten, F., Snounou, G., and White, N.J., *High-throughput ultrasensitive molecular techniques for quantifying low-density malaria parasitemias.* J Clin Microbiol, 2014. **52**(9): p. 3303-9.

250. Acestor, N., Cooksey, R., Newton, P.N., Menard, D., Guerin, P.J., Nakagawa, J., Christophel, E., Gonzalez, I.J., and Bell, D., *Mapping the aetiology of non-malarial febrile illness in Southeast Asia through a systematic review--terra incognita impairing treatment policies.* PLoS One, 2012. **7**(9): p. e44269.

251. Cowan, G., *Rickettsial diseases: the typhus group of fevers--a review.* Postgrad Med J, 2000. **76**(895): p. 269-72.

252. Carrara, V.I., Lwin, K.M., Phyo, A.P., Ashley, E., Wiladphaingern, J., Sriprawat, K., Rijken, M., Boel, M., McGready, R., Proux, S., Chu, C., Singhasivanon, P., White, N., and Nosten, F., *Malaria burden and artemisinin resistance in the mobile and migrant population on the Thai-Myanmar border, 1999-2011: an observational study.* PLoS Med, 2013. **10**(3): p. e1001398.

253. Imwong, M., Nguyen, T.N., Tripura, R., Peto, T.J., Lee, S.J., Lwin, K.M., Suangkanarat, P., Jeeyapant, A., Vihokhern, B., Wongsaen, K., Van Hue, D., Dong

le, T., Nguyen, T.U., Lubell, Y., von Seidlein, L., Dhorda, M., Promnarate, C., Snounou, G., Malleret, B., Renia, L., Keereecharoen, L., Singhasivanon, P., Sirithiranont, P., Chalk, J., Nguon, C., Hien, T.T., Day, N., White, N.J., Dondorp, A., and Nosten, F., *The epidemiology of subclinical malaria infections in South-East Asia: findings from cross-sectional surveys in Thailand-Myanmar border areas, Cambodia, and Vietnam.* Malar J, 2015. **14**: p. 381.

254. Watthanaworawit W, Turner P, Turner C, Dohnt M, Tanganuchitcharnchai A, Blacksell SD, and F, N., *Diagnostic Accuracy of Real-Time PCR for Dengue, Leptospirosis, and Scrub typhus Infections – A Field Evaluation*, in *EID 2010 – Thailand Conference on Emerging Infectious and Neglected Diseases. Amari Orchid Resort and Tower, Pattaya, Chonburi, Thailand*. 2010.

255. Banoo, S., Bell, D., Bossuyt, P., Herring, A., Mabey, D., Poole, F., Smith, P.G., Sriram, N., Wongsrichanalai, C., Linke, R., O'Brien, R., Perkins, M., Cunningham, J., Matsoso, P., Nathanson, C.M., Olliaro, P., Peeling, R.W., and Ramsay, A., *Evaluation of diagnostic tests for infectious diseases: general principles.* Nat Rev Microbiol, 2006. **4**(12 Suppl): p. S20-32.

256. Turner, P., Turner, C.L., Watthanaworawit, W., Carrara, V.I., Kapella, B.K., Painter, J., and Nosten, F.H., *Influenza in refugees on the Thailand-Myanmar border, May-October 2009.* Emerg Infect Dis, 2010. **16**(9): p. 1366-72.

257. Mouatcho, J.C. and Goldring, J.P., *Malaria rapid diagnostic tests: challenges and prospects.* J Med Microbiol, 2013. **62**(Pt 10): p. 1491-505.

258. Vaughn, D.W., Green, S., Kalayanarooj, S., Innis, B.L., Nimmannitya, S., Suntayakorn, S., Rothman, A.L., Ennis, F.A., and Nisalak, A., *Dengue in the early febrile phase: viremia and antibody responses.* J Infect Dis, 1997. **176**(2): p. 322-30.

259. Shu, P.Y., Chang, S.F., Kuo, Y.C., Yueh, Y.Y., Chien, L.J., Sue, C.L., Lin, T.H., and Huang, J.H., *Development of group- and serotype-specific one-step SYBR green I-based real-time reverse transcription-PCR assay for dengue virus.* J Clin Microbiol, 2003. **41**(6): p. 2408-16.

260. Emery, S.L., Erdman, D.D., Bowen, M.D., Newton, B.R., Winchell, J.M., Meyer, R.F., Tong, S., Cook, B.T., Holloway, B.P., McCaustland, K.A., Rota, P.A., Bankamp, B., Lowe, L.E., Ksiazek, T.G., Bellini, W.J., and Anderson, L.J., *Real-time reverse transcription-polymerase chain reaction assay for SARS-associated coronavirus.* Emerg Infect Dis, 2004. **10**(2): p. 311-6.

261. Suwanabun, N., Chouriyagune, C., Eamsila, C., Watcharapichat, P., Dasch, G.A., Howard, R.S., and Kelly, D.J., *Evaluation of an enzyme-linked immunosorbent assay in Thai scrub typhus patients.* Am J Trop Med Hyg, 1997. **56**(1): p. 38-43.

262. Richards, A.L., Rahardjo, E., Rusjdi, A.F., Kelly, D.J., Dasch, G.A., Church, C.J., and Bangs, M.J., *Evidence of Rickettsia typhi and the potential for murine typhus in Jayapura, Irian Jaya, Indonesia.* Am J Trop Med Hyg, 2002. **66**(4): p. 431-4.

263. Robinson, D.M., Brown, G., Gan, E., and Huxsoll, D.L., *Adaptation of a microimmunofluorescence test to the study of human Rickettsia tsutsugamuskh antibody.* Am J Trop Med Hyg, 1976. **25**(6): p. 900-5.

264. Qiagen. *QIAamp® DNA Mini and Blood Mini Handbook.* 2015 [cited 2016 February]; Fourth Edition:[Available from: https://www.qiagen.com/us/resources/resourcedetail?id=67893a91-946f-49b5-8033-394fa5d752ea.

265. Kamau, E., Tolbert, L.S., Kortepeter, L., Pratt, M., Nyakoe, N., Muringo, L., Ogutu, B., Waitumbi, J.N., and Ockenhouse, C.F., *Development of a highly sensitive genus-specific quantitative reverse transcriptase real-time PCR assay for*

detection and quantitation of plasmodium by amplifying RNA and DNA of the 18S rRNA genes. J Clin Microbiol. **49**(8): p. 2946-53.

266. Imwong, M., Nair, S., Pukrittayakamee, S., Sudimack, D., Williams, J.T., Mayxay, M., Newton, P.N., Kim, J.R., Nandy, A., Osorio, L., Carlton, J.M., White, N.J., Day, N.P., and Anderson, T.J., *Contrasting genetic structure in Plasmodium vivax populations from Asia and South America.* Int J Parasitol, 2007. **37**(8-9): p. 1013-22.

267. Anderson, T.J., Su, X.Z., Bockarie, M., Lagog, M., and Day, K.P., *Twelve microsatellite markers for characterization of Plasmodium falciparum from finger-prick blood samples.* Parasitology, 1999. **119 (Pt 2)**: p. 113-25.

268. Lubell, Y., Blacksell, S.D., Dunachie, S., Tanganuchitcharnchai, A., Althaus, T., Watthanaworawit, W., Paris, D.H., Mayxay, M., Peto, T.J., Dondorp, A.M., White, N.J., Day, N.P., Nosten, F., Newton, P.N., and Turner, P., *Performance of C-reactive protein and procalcitonin to distinguish viral from bacterial and malarial causes of fever in Southeast Asia.* BMC Infect Dis, 2015. **15**: p. 511.

269. Patel, U., Gandhi, G., Friedman, S., and Niranjan, S., *Thrombocytopenia in malaria.* J Natl Med Assoc, 2004. **96**(9): p. 1212-4.

270. Hanson, J., Phu, N.H., Hasan, M.U., Charunwatthana, P., Plewes, K., Maude, R.J., Prapansilp, P., Kingston, H.W., Mishra, S.K., Mohanty, S., Price, R.N., Faiz, M.A., Dondorp, A.M., White, N.J., Hien, T.T., and Day, N.P., *The clinical implications of thrombocytopenia in adults with severe falciparum malaria: a retrospective analysis.* BMC Med, 2015. **13**: p. 97.

271. Thwaites, G.E., Chau, T.T., Stepniewska, K., Phu, N.H., Chuong, L.V., Sinh, D.X., White, N.J., Parry, C.M., and Farrar, J.J., *Diagnosis of adult tuberculous meningitis by use of clinical and laboratory features.* Lancet, 2002. **360**(9342): p. 1287-92.

272. Wintrobe, M.M. and Greer, J.P., *Wintrobe's Clinical Hematology*. 12th ed. 2009: Wolters Kluwer Health/Lippincott Williams & Wilkins.

273. Chambers, T.L., *Forfar & Arneil's Textbook of Pediatrics: 6th edition.* Journal of the Royal Society of Medicine, 2004. **97**(2): p. 96-96.

274. Kliegman, R.M., Behrman, R.E., Jenson, H.B., and Stanton, B.M.D., *Nelson Textbook of Pediatrics*. 18th ed. 2007: Elsevier Health Sciences.

275. Watt, G., Jongsakul, K., and Suttinont, C., *Possible scrub typhus coinfections in Thai agricultural workers hospitalized with leptospirosis.* Am J Trop Med Hyg, 2003. **68**(1): p. 89-91.

276. Limkittikul, K., Brett, J., and L'Azou, M., *Epidemiological trends of dengue disease in Thailand (2000-2011): a systematic literature review.* PLoS Negl Trop Dis, 2014. **8**(11): p. e3241.

277. Mitaka, C., *Clinical laboratory differentiation of infectious versus non-infectious systemic inflammatory response syndrome.* Clin Chim Acta, 2005. **351**(1-2): p. 17-29.

278. Gatton, M.L. and Cheng, Q., *Evaluation of the pyrogenic threshold for Plasmodium falciparum malaria in naive individuals.* Am J Trop Med Hyg, 2002. **66**(5): p. 467-73.

279. Imwong, M., Stepniewska, K., Tripura, R., Peto, T.J., Lwin, K.M., Vihokhern, B., Wongsaen, K., von Seidlein, L., Dhorda, M., Snounou, G., Keereecharoen, L., Singhasivanon, P., Sirithiranont, P., Chalk, J., Nguon, C., Day, N.P., Nosten, F., Dondorp, A., and White, N.J., *Numerical Distributions of Parasite Densities During Asymptomatic Malaria.* J Infect Dis, 2016. **213**(8): p. 1322-9.

280. Congpuonga, K., Gilb, J.P., Bualombaic, P., and Kangchaingonea, Y., *Mixed-species malaria infection in high transmission areas of Thailand.* Asian Biomedicine, 2008. **2**(2): p. 117-21.

281. Chareonviriyaphap, T., Bangs, M.J., and Ratanatham, S., *Status of malaria in Thailand.* Southeast Asian J Trop Med Public Health, 2000. **31**(2): p. 225-37.

282. Siribal, S., Nakasiri, S., Looareesuwan, S., and Chavalitshewinkoon-Petmitr, P., *Identification of human malaria parasites and detection of mixed infection in Thai patients by nested PCR.* Southeast Asian J Trop Med Public Health, 2004. **35 Suppl 2**: p. 5-9.

283. Snounou, G. and White, N.J., *The co-existence of Plasmodium: sidelights from falciparum and vivax malaria in Thailand.* Trends Parasitol, 2004. **20**(7): p. 333-9.

284. Brown, M.G., Vickers, I.E., Salas, R.A., and Smikle, M.F., *Leptospirosis in suspected cases of dengue in Jamaica, 2002-2007.* Trop Doct, 2010. **40**(2): p. 92-4.

285. Seed, P., *DIAGT: Stata module to report summary statistics for diagnostic tests compared to true disease status*. 2001, Boston College Department of Economics: Chestnut Hill, MA, USA.

286. Landis, J.R. and Koch, G.G., *The measurement of observer agreement for categorical data.* Biometrics, 1977. **33**(1): p. 159-74.

287. Phommasone, K., Sengvilaipaseuth, O., de Lamballerie, X., Vongsouvath, M., Phonemixay, O., Blacksell, S.D., Newton, P.N., and Dubot-Peres, A., *Temperature and the Field Stability of a Dengue Rapid Diagnostic Test in the Tropics.* Am J Trop Med Hyg, 2015.

288. Teoh, B.T., Sam, S.S., Tan, K.K., Johari, J., Abd-Jamil, J., Hooi, P.S., and AbuBakar, S., *The Use of NS1 Rapid Diagnostic Test and qRT-PCR to Complement IgM ELISA for Improved Dengue Diagnosis from Single Specimen.* Sci Rep, 2016. **6**: p. 27663.

289. Lee, J., Kim, H.Y., Chong, C.K., and Song, H.O., *Development and clinical evaluation of a highly accurate dengue NS1 rapid test: from the preparation of a*

soluble NS1 antigen to the construction of an RDT. Diagn Microbiol Infect Dis, 2015.

290. Hunsperger, E.A., Yoksan, S., Buchy, P., Nguyen, V.C., Sekaran, S.D., Enria, D.A., Vazquez, S., Cartozian, E., Pelegrino, J.L., Artsob, H., Guzman, M.G., Olliaro, P., Zwang, J., Guillerm, M., Kliks, S., Halstead, S., Peeling, R.W., and Margolis, H.S., *Evaluation of commercially available diagnostic tests for the detection of dengue virus NS1 antigen and anti-dengue virus IgM antibody.* PLoS Negl Trop Dis, 2014. **8**(10): p. e3171.

291. Kim, Y.J., Yeo, S.J., Park, S.J., Woo, Y.J., Kim, M.W., Kim, S.H., Chang, I.A., Jeon, S.H., Park, B.J., Song, G.J., Lee, M.G., Kim, I.S., and Kim, Y.W., *Improvement of the diagnostic sensitivity of scrub typhus using a mixture of recombinant antigens derived from Orientia tsutsugamushi serotypes.* J Korean Med Sci, 2013. **28**(5): p. 672-9.

292. Silpasakorn, S., Srisamut, N., Ekpo, P., Zhang, Z., Chao, C.C., Ching, W.M., and Suputtamongkol, Y., *Development of New, Broadly Reactive, Rapid IgG and IgM Lateral Flow Assays for Diagnosis of Scrub Typhus.* Am J Trop Med Hyg, 2012. **87**(1): p. 148-52.

293. Gail, M.H., Sheehy, T., Cosentino, M., Pee, D., Diaz-Mayoral, N.A., Garcia-Closas, M., Caporaso, N.E., Pitt, K., and Ziegler, R.G., *Maximizing DNA yield for epidemiologic studies: no more buffy coats?* Am J Epidemiol, 2013. **178**(7): p. 1170-6.

294. McGready, R., Prakash, J.A., Benjamin, S.J., Watthanaworawit, W., Anantatat, T., Tanganuchitcharnchai, A., Ling, C.L., Tan, S.O., Ashley, E.A., Pimanpanarak, M., Blacksell, S.D., Day, N.P., Singhasivanon, P., White, N.J., Nosten, F., and Paris, D.H., *Pregnancy outcome in relation to treatment of murine typhus and scrub*

typhus infection: a fever cohort and a case series analysis. PLoS Negl Trop Dis, 2014. **8**(11): p. e3327.

295. Blacksell, S.D., Luksameetanasan, R., Kalambaheti, T., Aukkanit, N., Paris, D.H., McGready, R., Nosten, F., Peacock, S.J., and Day, N.P., *Genetic typing of the 56-kDa type-specific antigen gene of contemporary Orientia tsutsugamushi isolates causing human scrub typhus at two sites in north-eastern and western Thailand.* FEMS Immunol Med Microbiol, 2008. **52**(3): p. 335-42.

296. Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., and Wittwer, C.T., *The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments.* Clin Chem, 2009. **55**(4): p. 611-22.

297. Leeflang, M.M., Bossuyt, P.M., and Irwig, L., *Diagnostic test accuracy may vary with prevalence: implications for evidence-based diagnosis.* J Clin Epidemiol, 2009. **62**(1): p. 5-12.

298. Mabey, D., Peeling, R.W., Ustianowski, A., and Perkins, M.D., *Diagnostics for the developing world.* Nat Rev Microbiol, 2004. **2**(3): p. 231-40.

299. Morshed, M.G., Lee, M.K., Jorgensen, D., and Isaac-Renton, J.L., *Molecular methods used in clinical laboratory: prospects and pitfalls.* FEMS Immunol Med Microbiol, 2007. **49**(2): p. 184-91.

300. Tricou, V., Vu, H.T., Quynh, N.V., Nguyen, C.V., Tran, H.T., Farrar, J., Wills, B., and Simmons, C.P., *Comparison of two dengue NS1 rapid tests for sensitivity, specificity and relationship to viraemia and antibody responses.* BMC Infect Dis, 2010. **10**: p. 142.

301. Buathong, R., Hermann, L., Thaisomboonsuk, B., Rutvisuttinunt, W., Klungthong, C., Chinnawirotpisan, P., Manasatienkij, W., Nisalak, A., Fernandez, S., Yoon,

I.K., Akrasewi, P., and Plipat, T., *Detection of Zika Virus Infection in Thailand, 2012-2014.* Am J Trop Med Hyg, 2015. **93**(2): p. 380-3.

302. World Health Organization (WHO). *Zika Situation report*. 2016 6 October 2016 [cited 2017 13 January]; Available from: http://www.who.int/emergencies/zika-virus/situation-report/6-october-2016/en/.

303. Lanciotti, R.S., Kosoy, O.L., Laven, J.J., Velez, J.O., Lambert, A.J., Johnson, A.J., Stanfield, S.M., and Duffy, M.R., *Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007.* Emerg Infect Dis, 2008. **14**(8): p. 1232-9.

304. Chiu, C.Y., *Viral pathogen discovery.* Curr Opin Microbiol, 2013. **16**(4): p. 468-78.

11 Appendix 1 Case report form (CRF) of fever diagnostic study (FDS)

SMRU Fever Study (FDS)

Study code: |__|__|__|-|__|__|__|__| Clinic code: |__|__|__|-|__|__|__|__|__|

Sex: |__| M |__| F Site: |__|__|__|

Vital Signs

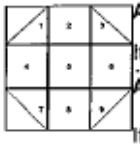
HR |__|__|__| per minute RR|__|__|__| per minute

Temp |__|__|.|__| °C |__|code of method (1.axillary 2.temporal 3.rectal 4.tympanic (ear) 5.other)

CRT |__|__|sec BP |__|__|__| / |__|__|__|

Symptoms and Signs

Fever	|__|No	|__|Yes	If Yes: |__|__| days
Headache	|__|No	|__|Yes	If Yes: |__|__| days
Pain when put chin on chest	|__|No	|__|Yes	If Yes: |__|__| days
Pain behind the eyes	|__|No	|__|Yes	If Yes: |__|__| days
Red eyes	|__|No	|__|Yes	If Yes: |__|__| days
Jaundice	|__|No	|__|Yes	If Yes: |__|__| days
Muscle pain	|__|No	|__|Yes	If Yes: |__|__| days
Joint pain	|__|No	|__|Yes	If Yes: |__|__| days
Cough	|__|No	|__|Yes	If Yes: |__|__| days
Constipation	|__|No	|__|Yes	If Yes: |__|__| days
Abdominal pain	|__|No	|__|Yes	If Yes: |__|__| days



If Yes where? |__| & |__| or Generalised |__|

Abnormal bleeding |__|No |__|Yes

If Yes: |__|__| days and what type |__| & |__|

1. vomiting blood 2. coughing up blood 3. blood from nose 4. bruising 5. bleeding from mouth 6. passing blood with stool 7. other

Skin rash |__|No |__|Yes

If Yes: |__|__| days and what type of rash |__| & |__|

1. petechiae 2. bruising 3. purpura 4. eschar 5. macular-papular rash 6. rose spots 7. other

Other symptoms |__|No |__|Yes

If Yes: |__|__| days Describe _____

Lymph nodes palpable |__|No |__|Yes If Yes where: |__|__|

1.cervical 2. axillary 3. groin 4. generalised

Malaria smear |__|Positive |__|Negative If Positive, parasitaemia: _____

Malaria RDT |__|Pf |__|P other sp. |__|Negative **RDT:** OptiMAL-IT / Paracheck / Other_____

Suspected diagnosis: _____ **Treatment:**_____

Investigations

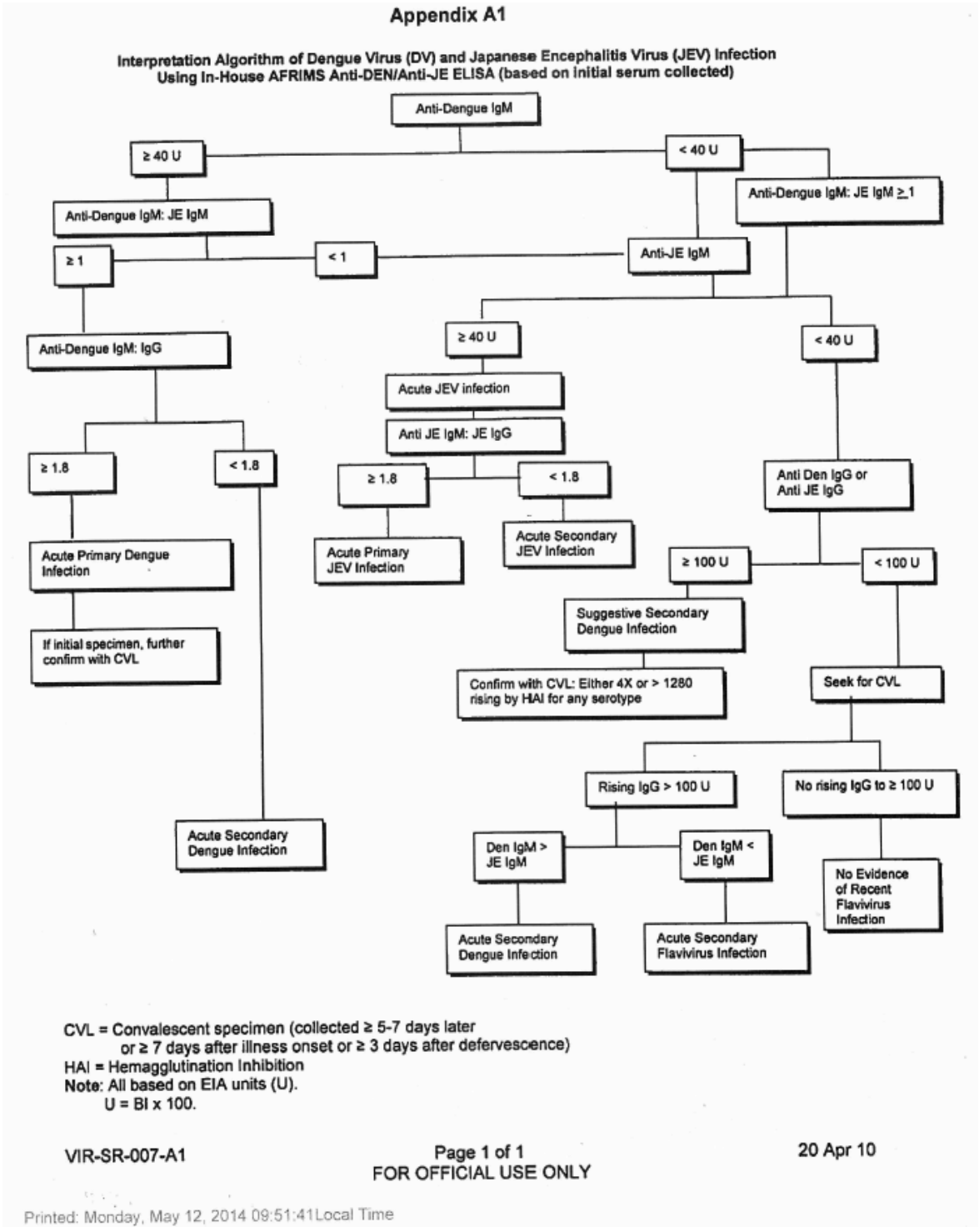
	Child (aged 5-17y)	**Adult (aged ≥18y)**
EDTA blood (CBC, CRP)	|__| 3ml	|__| 6ml
Venous blood (for serum)	|__| 3ml	|__| 5ml
Blood culture	|__| 2ml (BacT/ALERT PF bottle)	|__| 5ml (BacT/ALERT FA bottle)

Expected date of follow up blood sample |__|__|:|__|__|:|__|__|

Completed by: _____ **Date:** |__|__|:|__|__|:|__|__| [V1.2; 31st January 2011]

12 Appendix 2 Interpretation algorithm of dengue virus and Japanese encephalitis virus infection using in-house AFRIMS ELISA (AFRIMS SOP VIR-SR-007).

Permission from Ananda Nisalak, AFRIMS, Bangkok, Thailand



13 Appendix 3 Estimated cost and result turnaround time for alternative diagnostic tests used in the study

Diagnostic tests	Cost per test THB (USD)[a]	Result turnaround time
Dengue		
1. rRT-PCR	200 (5.9)	5 hours
Plus nucleic acid extraction	*300 (8.8)*	*2 hours*
Plus internal control assay	*200 (5.9)*	-[b]
2. SD BIOLINE Dengue Duo NS1, IgM/IgG ICT (Standard diagnostic, Inc.)	450 (13.2)	15 minutes
Leptospirosis		
3. 16S *rRNA* qPCR	200 (5.9)	3 hours
Plus nucleic acid extraction	*300 (8.8)*	*2 hours*
Plus internal control assay	*200 (5.9)*	-[b]
Scrub typhus		
4. 47kDa qPCR[c]	200 (5.9)	3 hours
Plus nucleic acid extraction	*300 (8.8)*	*4 hours*
Plus internal control assay	*200 (5.9)*	-[b]
5. SD BIOLINE Scrub typhus IgM ICT (Standard diagnostic, Inc.)	N/A[d]	15 minutes
Murine typhus and other *Rickettsia* spp.		
6. 17kDa qPCR[c]	200 (5.9)	3 hours
7. *ompB* qPCR[c]	200 (5.9)	3 hours
8. Sequencing (Macrogen)[e]	170.50 (5.0)	3-5 days
Plus nested PCR, gel electrophoresis and PCR product purification	*200 (5.9)*	*2 days*
Plus shipping	*852.25 (25)*	-[f]
Invasive bacterial infection		
9. Blood culture	200-400 (5.9-11.7)	1-7 days

[a]1 USD = 34.09 THB, [b]Internal control assay was performed together with the target assays, [c]Nucleic acid extraction and internal control assay were performed only once for scrub typhus, murine typhus and other *Rickettsia* spp. qPCRs, [d]Not commercially available-supported by the Standards diagnostics, Inc (free of charge), [e]sent to Macrogen, Republic of Korea, [f]3-5 days including testing.

14 Appendix 4 Publications resulting from this thesis

Watthanaworawit, W., Turner, P., Turner, C., Tanganuchitcharnchai, A., Richards, A.L., Bourzac, K.M., Blacksell, S.D., and Nosten, F., *A Prospective Evaluation of Real-Time PCR Assays for the Detection of Orientia tsutsugamushi and Rickettsia spp. for Early Diagnosis of Rickettsial Infections during the Acute Phase of Undifferentiated Febrile Illness.* Am J Trop Med Hyg, 2013. **89**(2): p. 308-10.

Watthanaworawit, W., Turner, P., Turner, C., Tanganuchitcharnchai, A., Jintaworn, S., Hanboonkunupakarn, B., Richards, A.L., Day, N.P., Blacksell, S.D., and Nosten, F., *Diagnostic Accuracy Assessment of Immunochromatographic Tests for the Rapid Detection of Antibodies Against Orientia tsutsugamushi Using Paired Acute and Convalescent Specimens.* Am J Trop Med Hyg, 2015. 93(6): p. 1168-71.

Am. J. Trop. Med. Hyg., 89(2), 2013, pp. 308–310
doi:10.4269/ajtmh.12-0600

Short Report: A Prospective Evaluation of Real-Time PCR Assays for the Detection of *Orientia tsutsugamushi* and *Rickettsia* spp. for Early Diagnosis of Rickettsial Infections during the Acute Phase of Undifferentiated Febrile Illness

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Abstract. One hundred and eighty febrile patients were analyzed in a prospective evaluation of *Orientia tsutsugamushi* and *Rickettsia* spp. real-time polymerase chain reaction (PCR) assays for early diagnosis of rickettsial infections. By paired serology, 3.9% (7 of 180) and 6.1% (11 of 180) of patients were confirmed to have acute scrub or murine typhus, respectively. The PCR assays for the detection of *O. tsutsugamushi* and *Rickettsia* spp. had high specificity (99.4% [95% confidence interval (CI): 96.8–100] and 100% [95% CI: 97.8–100], respectively). The PCR results were also compared with immunoglobulin M (IgM) immunofluorescence assay (IFA) on acute sera. For *O. tsutsugamushi*, PCR sensitivity was twice that of acute specimen IgM IFA (28.6% versus 14.3%; McNemar's $P = 0.3$). For *Rickettsia* spp., PCR was four times as sensitive as acute specimen IgM IFA (36.4% versus 9.1%; $P = 0.08$), although this was not statistically significant. Whole blood and buffy coat, but not serum, were acceptable specimens for these PCRs. Further evaluation of these assays in a larger prospective study is warranted.

INTRODUCTION

Scrub and murine typhus, caused by *Orientia tsutsugamushi* and *Rickettsia typhi*, respectively, are important acute febrile illnesses in Thailand.[1,2] Clinical diagnosis is difficult because early symptoms are similar to other common infections such as dengue, leptospirosis, and malaria.[3] Laboratory confirmation is also difficult, conventionally requiring either culture of the infectious agent in cell monolayers at biosafety level-3,[4] or paired serology (indirect immunofluorescence assay [IFA]) for the detection of rising antibody titers.[5]

MATERIALS AND METHODS

We prospectively evaluated two real-time polymerase chain reaction (PCR) assays for the rapid diagnosis of rickettsial infections using several blood fractions. These assays were designed for use on the JBAIDS instrument (Joint Biological Agent Identification and Diagnostic System; BioFire Diagnostics, Inc. (formerly Idaho Technology), Salt Lake City, UT): a ruggedized, field-portable real-time PCR system.

Specimens from 180 patients recruited into a study of fever of unknown origin on the Thailand-Myanmar border were included in the evaluation. Consenting patients' ≥ 5 years of age, with a febrile illness (≥ 38°C) of ≤ 7 days duration and a negative malaria test, were eligible for inclusion. Venous blood (EDTA tube) was collected for PCR at enrollment and serum was collected at enrollment and Day 14 for IFA analyses. Blood specimens were aliquoted and stored at –80°C before testing. This study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Thailand (MUTM 2011-008-01) and Oxford Tropical Research Ethics Committee (OXTREC 42-10).

To compare the impact of use of different blood fractions in these PCRs, DNA was extracted from whole blood (adult patients only), buffy coat (all patients), and selected acute serum specimens (described below). Eight hundred microliters of whole blood and serum were extracted using the IT 1-2-3 Platinum Path purification kit (BioFire Diagnostics, Inc.) and 200 μL of buffy coat was extracted using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Elution volumes were 200 μL (whole blood/buffy coat) and 100 μL (serum).

Two JBAIDS-based TaqMan probe-based real-time PCR assays were designed for the specific detection of *O. tsutsugamushi* (47-kDa outer membrane protein gene) or *Rickettsia* spp. (17-kDa antigen gene). Primer and probe sequences for 17-kDa gene were modified slightly from previously published assays (see Reference 6) and optimized for use on the JBAIDS platform (R17KF_4: 5′-AAA CAA GGK CAN GGH ACA CTT CTT G-3′, R17KR: 5′-AAG TAA TGC RCC TAC ACC TAC TC-3′, and RProbeV2: 5′-6FAM-CCG AAT TGA GAA CCA AGT AAT GC-TAMRA-3′). The limit of detection was determined by testing the lowest level of spiked template that could be detected in at least 95% of specimens (i.e., 19 out of 20 replicates) and found to be 25 copies of target template per reaction. Both target assays were multiplexed with an internal inhibition control. The DNA extracts (20 μL/assay) were added to the freeze-dried reagent tubes along with 20 μL of reconstitution buffer and immediately tested in duplicate (individual reaction volume 20 μL; DNA template volume 10 μL/reaction) on the JBAIDS instrument. Positive and negative controls were included in each PCR run. The JBAIDS instrument software automatically determined the specific operation of the instrument, i.e. times, temperatures, and number of PCR cycles, and determined a result for the duplicate reactions. Whole blood and buffy coat specimens were tested for all adult patients. For children, only buffy coat fractions were tested because there was insufficient whole blood/serum to be tested. If an adult patient tested positive for the *O. tsutsugamushi* or *Rickettsia* assay in whole blood or buffy coat, the corresponding serum

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specimen was then also tested. The PCR was repeated for specimens yielding uncertain results (discordant results between duplicate reactions) on first testing.

For comparison, three alternate rickettsial real-time PCR assays were run on buffy coat specimens, using the same DNA extracts as for the JBAIDS-based PCRs. These PCRs targeted 47-kDa outer membrane protein gene of *O. tsutsugamushi*,[6] 17-kDa antigen gene of *Rickettsia* spp.,[7] and *R. typhi* ompB,[8] as previously described. A human RNaseP gene PCR was used as an internal control to confirm the absence of PCR inhibition and to monitor extraction efficiency.[9] One microliter of DNA template was added into each 25 μL PCR reaction. The PCR was done on an ABI 7500 Fast instrument (Life Technologies, Grand Island, NY).

All statistical data analyses were perform using STATA/SE 10.1 (StataCorp, College Station, TX). Diagnostic accuracy of the PCRs was calculated by comparing PCR results to the paired sera IFA results in 2×2 cross tabulation using the "diagt" routine.[10] The McNemar test was used to compare test sensitivities. Binomial exact confidence intervals were calculated throughout.

RESULTS

Using a ≥ 4-fold rise in IFA immunoglobulin M (IgM) antibody titers between the paired acute and convalescent serum specimens to define acute infection,[11] 6.1% (11 of 180) of the patients were diagnosed with acute murine typhus and 3.9% (7 of 180) were with acute scrub typhus. Using a cut-off titer of 400 as evidence of acute infection as previously described,[12] the sensitivity and specificity of acute specimen IgM IFA were 14.3% (95% confidence interval [CI]: 0.4–57.9) and 97.7% (95% CI: 94.2–99.4) for scrub typhus, 9.1% (95% CI: 0.2–41.3) and 99.4% (95% CI: 96.7–100) for murine typhus (raw data not shown), respectively.

One hundred and four whole blood fractions and 180 buffy coat fractions were tested by JBAIDS PCR. Three and six corresponding serum fractions were tested for the *O. tsutsugamushi* and *Rickettsia* assay, respectively. The overall positivity of the *O. tsutsugamushi* and *Rickettsia* assays was 1.7% (3 of 180) and 2.2% (4 of 180), respectively, when any of the blood fractions gave a positive result.

The specificity of *O. tsutsugamushi* assay was 99.0% (95% CI: 94.6–100) using whole blood, 99.4% (95% CI: 96.8–100) using buffy coat, and 99.4% (95% CI: 96.8–100) when the results of all blood fractions were combined. The sensitivity of the assay using whole blood, buffy coat, and combined blood fractions was 50.0% (95% CI: 6.8–93.2), 28.6% (95% CI: 3.7–71.0), and 28.6% (95% CI: 3.7–71.0), respectively. Serum fractions did not correctly identify either of the patients with positive whole blood or buffy coat fractions. A total of seven patients had serologically confirmed acute scrub typhus infection by paired IgM IFA. Of these, three patients were children and only the buffy coat fraction was tested by PCR, none of which gave positive results. Of the four adult patients, two were positive by PCR in both whole blood and buffy coat fractions, but were negative in the serum fraction. There was perfect correlation between results obtained from whole blood and buffy coat fractions. One additional adult patient who had no evidence of acute scrub typhus infection by paired IgM IFA was positive for PCR in all blood fractions.

Table 1

Comparison of two PCR assays for detection of *Orientia tsutsugamushi* DNA from blood*

	O. tsutsugamushi PCR (JBAIDS instrument)		47 kDa PCR (ABI instrument)	
Paired serology result (IgM IFA)	Negative	Positive	Negative	Positive
Not scrub typhus	172	1	172	1
Acute scrub typhus	5	2	5	2

*PCR = polymerase chain reaction; IFA = immunofluorescence assay; IgM = immunoglobulin M.

Table 2

Comparison of three PCR assays for detection of *Rickettsia* spp. DNA from blood*

	Rickettsia spp. PCR (JBAIDS instrument)			17 kDa PCR (ABI instrument)		ompB PCR (ABI instrument)	
Paired serology result (IgM IFA)	Negative	Positive	Uncertain	Negative	Positive	Negative	Positive
Not murine typhus	168	0	1	169	0	169	0
Acute murine typhus	8	3	0	9	2	9	2

*PCR = polymerase chain reaction; IgM = immunoglobulin M; IFA = immunofluorescence assay.

For *Rickettsia* assay, the specificity was 100% in all blood fractions, although as a consequence of the small number of serum specimens tested ($N = 6$), the 95% CI for the serum fraction result was wide (2.5–100). The sensitivity of the assay using whole blood, buffy coat, serum, and combined blood fractions was 30.0% (95% CI: 6.7–65.2), 27.3% (95% CI: 6.0–61.0), 40.0% (95% CI: 5.3–85.3), and 36.4% (95% CI: 10.9–69.2), respectively. A total of 11 patients had serologically confirmed positive acute murine typhus infection by paired IgM IFA. One patient was a child and only buffy coat fraction was tested by PCR, which had a negative result. Of the 10 adult patients, four patients were positive by PCR in whole blood and/or buffy coat fractions. There was poor correlation between the results obtained from whole blood and buffy coat fractions and the corresponding serum fraction.

For *O. tsutsugamushi*, PCR sensitivity was twice that of acute specimen IgM IFA (28.6% [95% CI: 3.7–71.0] versus 14.3% [95% CI: 0.4–57.9]; McNemar's $P = 0.3$). For *Rickettsia* spp., PCR was four times as sensitive as acute specimen IgM IFA (36.4% [95% CI: 10.9–69.2] versus 9.1% [95% CI: 0.2–41.3]; $P = 0.08$).

The comparison of five PCR assays for detection of *O. tsutsugamushi* and *Rickettsia* spp. DNA from blood are shown in Tables 1 and 2. Both *O. tsutsugamushi* PCR and 47-kDa PCR showed perfect concordant results; 1.67% (3 of 180) were positive for both PCRs (Table 1). For *Rickettsia* spp. PCR, 1.67% (3 of 180) was positive. One uncertain result was considered negative because there was no evidence for acute murine typhus by reference test (Table 2). However this result could represent a true infection by another spotted-fever group *Rickettsia* sp. 1.11% (2 of 180) were positive for 17-kDa PCR and the same specimens (1.11% [2 of 180]) were confirmedly positive by ompB PCR.

CONCLUSION

We found that both of the *O. tsutsugamushi* and *Rickettsia* assays had high specificity and there was good correlation with the alternate PCR assays. The clinical sensitivity was low but

PCR was more sensitive than acute specimen IgM IFA. It is probable that a combination of both molecular and antibody-based detection assays would be the ideal panel to adequately cover all diagnostic window periods and might allow confirmation of infection quickly enough to be useful for patient management.[13,14] A further evaluation of these real-time PCR assays in a larger prospective study is warranted.

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Disclaimer: The authors have no personal conflicts of interest. The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, or the U.S. Government. As an employee of the U.S. Government (ALR) this work was prepared as part of my official duties and therefore under Title 17 USC paragraph 105 copyright protection is not available.

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REFERENCES

1. Suttinont C, Losuwanaluk K, Niwatayakul K, Hoontrakul S, Intaranongpai W, Silpasakorn S, Suwancharoen D, Panlar P, Saisongkorh W, Rolain JM, Raoult D, Suputtamongkol Y, 2006. Causes of acute, undifferentiated, febrile illness in rural Thailand: results of a prospective observational study. *Ann Trop Med Parasitol 100:* 363–370.
2. McGready R, Ashley EA, Wuthiekanun V, Tan SO, Pimanpanarak M, Viladpai-Nguen SJ, Jesadapanpong W, Blacksell SD, Peacock SJ, Paris DH, Day NP, Singhasivanon P, White NJ, Nosten F, 2010. Arthropod borne disease: the leading cause of fever in pregnancy on the Thai-Burmese border. *PLoS Negl Trop Dis 4:* e888.
3. Ellis RD, Fukuda MM, McDaniel P, Welch K, Nisalak A, Murray CK, Gray MR, Uthaimongkol N, Buathong N, Sriwichai S, Phasuk R, Yingyuen K, Mathavarat C, Miller RS, 2006. Causes of fever in adults on the Thai-Myanmar border. *Am J Trop Med Hyg 74:* 108–113.
4. Luksameetanasan R, Blacksell SD, Kalambaheti T, Wuthiekanun V, Chierakul W, Chueasuwanchai S, Apiwattanaporn A, Stenos J, Graves S, Peacock SJ, Day NP, 2007. Patient and sample-related factors that effect the success of *in vitro* isolation of *Orientia tsutsugamushi. Southeast Asian J Trop Med Public Health 38:* 91–96.
5. Blacksell SD, Bryant NJ, Paris DH, Doust JA, Sakoda Y, Day NP, 2007. Scrub typhus serologic testing with the indirect immunofluorescence method as a diagnostic gold standard: a lack of consensus leads to a lot of confusion. *Clin Infect Dis 44:* 391–401.
6. Jiang J, Chan TC, Temenak JJ, Dasch GA, Ching WM, Richards AL, 2004. Development of a quantitative real-time polymerase chain reaction assay specific for *Orientia tsutsugamushi*. *Am J Trop Med Hyg 70:* 351–356.
7. Wright CL, Nadolny RM, Jiang J, Richards AL, Sonenshine DE, Gaff HD, Hynes WL, 2011. *Rickettsia parkeri* in gulf coast ticks, southeastern Virginia, USA. *Emerg Infect Dis 17:* 896–898.
8. Henry KM, Jiang J, Rozmajzl PJ, Azad AF, Macaluso KR, Richards AL, 2007. Development of quantitative real-time PCR assays to detect *Rickettsia typhi* and *Rickettsia felis*, the causative agents of murine typhus and flea-borne spotted fever. *Mol Cell Probes 21:* 17–23.
9. Emery SL, Erdman DD, Bowen MD, Newton BR, Winchell JM, Meyer RF, Tong S, Cook BT, Holloway BP, McCaustland KA, Rota PA, Bankamp B, Lowe LE, Ksiazek TG, Bellini WJ, Anderson LJ, 2004. Real-time reverse transcription-polymerase chain reaction assay for SARS-associated coronavirus. *Emerg Infect Dis 10:* 311–316.
10. Seed P, 2001. *DIAGT: Stata Module to Report Summary Statistics for Diagnostic Tests Compared to True Disease Status*. Boston, MA: Boston College Department of Economics.
11. Blacksell SD, Jenjaroen K, Phetsouvanh R, Tanganuchitcharnchai A, Phouminh P, Phongmany S, Day NP, Newton PN, 2010. Accuracy of rapid IgM-based immunochromatographic and immunoblot assays for diagnosis of acute scrub typhus and murine typhus infections in Laos. *Am J Trop Med Hyg 83:* 365–369.
12. Coleman RE, Sangkasuwan V, Suwanabun N, Eamsila C, Mungviriya S, Devine P, Richards AL, Rowland D, Ching WM, Sattabongkot J, Lerdthusnee K, 2002. Comparative evaluation of selected diagnostic assays for the detection of IgG and IgM antibody to *Orientia tsutsugamushi* in Thailand. *Am J Trop Med Hyg 67:* 497–503.
13. Paris DH, Blacksell SD, Nawtaisong P, Jenjaroen K, Teeraratkul A, Chierakul W, Wuthiekanun V, Kantipong P, Day NP, 2011. Diagnostic accuracy of a loop-mediated isothermal PCR assay for detection of *Orientia tsutsugamushi* during acute scrub typhus infection. *PLoS Negl Trop Dis 5:* e1307.
14. Koh GC, Maude RJ, Paris DH, Newton PN, Blacksell SD, 2010. Diagnosis of scrub typhus. *Am J Trop Med Hyg 82:* 368–370.

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Diagnostic Accuracy Assessment of Immunochromatographic Tests for the Rapid Detection of Antibodies against *Orientia tsutsugamushi* Using Paired Acute and Convalescent Specimens

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Abstract. We assessed the diagnostic accuracy of two immunochromatographic tests (ICTs), the Access Bio *CareStart* Scrub Typhus test (Somerset, NJ) (IgM), and the SD BIOLINE Tsutsugamushi test (Kyonggi-do, Republic of Korea) (IgG, IgM, or IgA) compared with indirect immunofluorescence assay (IFA) and real-time PCR results as reference tests using 86 paired acute and convalescent specimens from febrile patients. The sensitivity and specificity of the *CareStart* test were 23.3% (95% confidence interval [CI]: 11.8–38.6) and 81.4% (95% CI: 66.6–91.6), respectively, for acute specimens and 32.6% (95% CI: 19.1–48.5) and 79.1% (95% CI: 64.0–90.0), respectively, for convalescent specimens. For the SD BIOLINE test, sensitivity and specificity were 20.9% (95% CI: 10.0–36.0) and 74.4% (95% CI: 58.8–86.5), respectively, for acute specimens and 76.7% (95% CI: 61.4–88.2) and 76.7% (95% CI: 61.4–88.2), respectively, for convalescent specimens. The poor sensitivity obtained for both ICTs during this study when performed on acute specimens highlights the difficulties in prompt diagnosis of scrub typhus.

INTRODUCTION

Scrub typhus, caused by *Orientia tsutsugamushi*, is an important cause of acute undifferentiated febrile illness in Thailand[1,2] and a common cause of fever on the Thailand–Myanmar border.[3,4] Clinical manifestations are similar to other causes of fever such as dengue, leptospirosis, and malaria, making clinical diagnosis difficult.[4] Laboratory confirmation relies on isolation of the organism at biosafety level 3 or paired serology (indirect immunofluorescence assay [IFA]) or on a combination of tests, that is, in vitro isolation, IFA, and polymerase chain reaction (PCR),[5–7] which are expensive, time consuming, and do not provide results in time to inform patient management. Several groups have developed and assessed the clinical utility of rapid bedside diagnostic tests for early diagnosis of this infection to assist patient management.[8–11] However, either diagnostic accuracy or commercial availability is limited. We evaluated the performance characteristics of two immunochromatographic tests (ICTs), the SD BIOLINE Tsutsugamushi (Standard Diagnostics, Inc., Kyonggi-do, Republic of Korea) test and the *CareStart* Scrub Typhus test (Access Bio, Inc., Somerset, NJ) using paired acute and convalescent serum specimens from patients with undifferentiated febrile illness.

MATERIALS AND METHODS

From a recent fever study in northwest Thailand, 86 participants were retrospectively selected for the current evaluation: 43 were confirmed to have acute scrub typhus infection and 43 patients were confirmed as not having acute scrub typhus infection by the detection of specific IgM antibody by IFA and real-time PCR assay targeting the *O. tsutsugamushi* 47-kDa outer membrane protein gene.[12] Acute scrub typhus was defined as 1) ≥ 4-fold increase in IFA IgM titer, 2) seroconversion, 3) a high static titer of ≥ 1:25,600 between acute and convalescent specimens, and/or 4) PCR positive in the acute specimen. All specimens were stored at −80°C before testing. The study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Thailand (MUTM 2011-008-01) and Oxford Tropical Research Ethics Committee (OXTREC 42-10).

Two ICTs were assessed: the *CareStart* Scrub Typhus test for the detection of IgM antibody against *O. tsutsugamushi* (not currently commercially available) and the SD BIOLINE Tsutsugamushi test for the detection of IgG, IgM, or IgA antibodies to *O. tsutsugamushi*, which uses a recombinant *O. tsutsugamushi* (strains Kato, Karp, and Gilliam) surface antigen (available for purchase in Thailand for approximately 150 THB/US$ 4.6 /test and has the Conformité Européénne [CE] marking). Both ICTs were performed on both acute and convalescent specimens following the manufacturer's instructions. In brief, for the *CareStart* test, 10 μL serum was added to the test devices followed by one drop of assay buffer (40 μL). The results were read at 10 minutes. For the SD BIOLINE test, 10 μL serum was added to the test devices followed by three drops of assay diluent. The results of the tests were read at 15 minutes. Both tests had two lines, a test line "T" and a control line "C." An absence of "C" line indicated an invalid result. The results of the tests were read by three independent readers, and the majority result was used for the final interpretation.

All statistical analyses were calculated using STATA/SE 10.1 (StataCorp., College Station, TX). Diagnostic accuracy of the tests was calculated by comparing the ICT results with the reference (composite IFA and PCR) results. A 2 × 2 table was constructed, in which the reference results were cross-tabulated with the ICT results to define the rate of true-positive, true-negative, false-positive, and false-negative results. The sensitivity, specificity, positive predictive value, and negative predictive value with 95% confidence intervals (CIs) were calculated

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using the "diagt" routine.[13] Kappa values were generated to determine the level of interoperator variation in the reading of the ICT test results.[14]

RESULTS AND DISCUSSION

Of the 86 patients tested, 68.6% (59/86) were male. The median age was 20 years (interquartile range [IQR]: 14–35 years), median temperature at presentation was 38.6°C (IQR: 38.2–39.1°C), median duration of fever at the time of presentation was 2 days (IQR: 2–3 days), and the median interval between obtaining initial acute-phase specimens and convalescent specimens was 14 days (range: 11–30 days). The performance characteristics of the *CareStart* Scrub Typhus test and the SD BIOLINE Tsutsugamushi test compared with the results of the reference tests are shown in Table 1. The sensitivity of the *CareStart* test was low for both acute and convalescent specimens (23.3% [95% CI: 11.8–38.6] and 32.6% [95% CI: 19.1–48.5], respectively). The specificities of the *CareStart* test using both acute and convalescent specimens were 81.4% (95% CI: 66.6–91.6) and 79.1% (95% CI: 64.0–90.0), respectively. Both the sensitivity and specificity of the *CareStart* test were much lower in this study than in the study previously reported by Blacksell and others,[9] which found the sensitivity to be 96.8% and the specificity to be 93.3% for acute-phase specimens. This may reflect the fact that our patients presented earlier in the course of their illness, when there is an absence of antibodies, with a median of 2 days (IQR: 2–3 days) of fever, compared with 8 days (range: 7–10 days) in the study of Blacksell and others. This result is consistent with the temporal antibody response for scrub typhus infectious.[15]

For the SD BIOLINE test, the sensitivity was higher for convalescent specimens (76.7% [95% CI: 61.4–88.2]) compared with acute specimens (20.9% [95% CI: 10.0–36.0]). The specificity of the test was similar when performed on acute and convalescent specimens (74.4% [95% CI: 58.8–86.5] and 76.7% [95% CI: 61.4–88.2], respectively). The sensitivity and specificity of the test obtained in this study were lower than those that the company claimed in the product insert (99% and 96%, respectively), although, the timing of blood collection was not mentioned. It is difficult to conclude, because of the limitations of the test, whether IgG, IgM, or IgA play a role in the positive result and whether the specificity of the test is compromised due to the detection of past infections.

Both ICTs tested in this study demonstrated very good interoperator agreement among three readers (Table 1). The SD BIOLINE test demonstrated better kappa values (0.98 and 0.98 for acute and convalescent specimens, respectively) compared with those of the *CareStart* test (0.84 and 0.85 for acute and convalescent specimens, respectively).

Given the small sample size of this evaluation, the 95% CI around the calculated sensitivities and specificities are wide. However, from these preliminary findings, both tests had poor sensitivity when performed on acute specimens taken early in the course of infection; however, this was improved for convalescent specimens. Consequently, both tests when performed at the acute phase of infection are not likely to be useful for patient management. Individual IgM or IgG antibody-based tests taken at appropriate time points post-onset of fever could be performed, which may give a clearer answer and aid the differentiation of past and current infections. In addition, there were four scrub typhus patients who had low IFA IgM titers on acute specimens (two were less than 100, one at 100, and one at 200, rising to 6,400, 12,800, 12,800, and 25,600 in convalescent specimens, respectively) but were positive by 47-kDa real-time PCR assay, none of whom were positive by either ICTs tested using acute specimens. This emphasizes the usefulness of nucleic acid detection during acute phase of scrub typhus infection. The eschar PCR could be useful for early diagnosis of scrub typhus. It is more likely to give a positive result than blood, especially, when patient has already been treated with antibiotics.[16] In our study, blood specimens were collected before antibiotics were started, and none of the patients in this sample set presented with eschar at the enrollment. It should be noted that the use of IFA alone as the gold standard test has limitations, and it is far from a perfect gold standard as standard IFA slides are not available, there is no consensus on cutoff titers, and difficulties with cross-reactivity and subjective endpoint

TABLE 1

The performance characteristics of *CareStart* Scrub Typhus assay for detection of IgM antibody and SD BIOLINE Tsutsugamushi assay for detection of IgG, IgM, or IgA antibodies using acute and convalescent specimens compared with the results of reference tests*

Specimen timing and diagnosis	Positive	Negative	% Sensitivity (95% CI)	% Specificity (95% CI)	% PPV (95% CI)	% NPV (95% CI)	Kappa value
CareStart Scrub Typhus IgM (N = 86)							
Acute							
Acute scrub typhus	10	33	23.3 (11.8–38.6)	81.4 (66.6–91.6)	55.6 (30.8–78.5)	51.5 (39.0–63.8)	0.84
Not acute scrub typhus†	8	35					
Convalescent							
Acute scrub typhus	14	29	32.6 (19.1–48.5)	79.1 (64.0–90.0)	60.9 (38.5–80.3)	54.0 (40.9–66.6)	0.85
Not acute scrub typhus	9	34					
SD BIOLINE Tsutsugamushi IgG, IgM, or IgA (N = 86)							
Acute							
Acute scrub typhus	9	34	20.9 (10.0–36.0)	74.4 (58.8–86.5)	45.0 (23.1–68.5)	48.5 (36.0–61.1)	0.98
Not acute scrub typhus	11	32					
Convalescent							
Acute scrub typhus	33	10	76.7 (61.4–88.2)	76.7 (61.4–88.2)	76.7 (61.4–88.2)	76.7 (61.4–88.2)	0.98
Not acute scrub typhus	10	33					

CI = confidence interval; IgM = immunoglobulin M; PPV = positive predictive value; NPV = negative predictive value.
*Immunofluorescence assay (IFA) IgM antibody and 47-kDa real-time polymerase chain reaction (PCR).
†Five cases of dengue, one Japanese encephalitis, 10 leptospirosis, 11 malaria, and 16 undiagnosed.

(reader variability). Therefore using it as the reference test could have affected negatively on the performance results of the novel tests.[6,17] However, the appropriate gold standard for scrub typhus remains elusive.

This study illustrates the limitations of two ICTs for the timely diagnosis of scrub typhus, highlighting the need to further investigate the utility of other diagnostic methodologies, for example, antigen- or nucleic acid-based detection (which may be more useful for diagnosing scrub typhus, especially for patients presenting to the clinic early in the course of their illness, when antibody responses have not yet developed) and/or combination of antigen/nucleic acid with antibody-based rapid detection that may improve the diagnostic accuracy for early diagnosis.[5,18]

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REFERENCES

1. Suttinont C, Losuwanaluk K, Niwatayakul K, Hoontrakul S, Intaranongpai W, Silpasakorn S, Suwancharoen D, Panlar P, Saisongkorh W, Rolain JM, Raoult D, Suputtamongkol Y, 2006. Causes of acute, undifferentiated, febrile illness in rural Thailand: results of a prospective observational study. *Ann Trop Med Parasitol 100:* 363–370.
2. Coleman RE, Sangkasuwan V, Suwanabun N, Eamsila C, Mungviriya S, Devine P, Richards AL, Rowland D, Ching WM, Sattabongkot J, Lerdthusnee K, 2002. Comparative evaluation of selected diagnostic assays for the detection of IgG and IgM antibody to *Orientia tsutsugamushi* in Thailand. *Am J Trop Med Hyg 67:* 497–503.
3. McGready R, Ashley EA, Wuthiekanun V, Tan SO, Pimanpanarak M, Viladpai-Nguen SJ, Jesadapanpong W, Blacksell SD, Peacock SJ, Paris DH, Day NP, Singhasivanon P, White NJ, Nosten F, 2010. Arthropod borne disease: the leading cause of fever in pregnancy on the Thai–Burmese border. *PLoS Negl Trop Dis 4:* e888.
4. Ellis RD, Fukuda MM, McDaniel P, Welch K, Nisalak A, Murray CK, Gray MR, Uthaimongkol N, Buathong N, Sriwichai S, Phasuk R, Yingyuen K, Mathavarat C, Miller RS, 2006. Causes of fever in adults on the Thai–Myanmar border. *Am J Trop Med Hyg 74:* 108–113.
5. Blacksell SD, Paris DH, Chierakul W, Wuthiekanun V, Teeratakul A, Kantipong P, Day NP, 2012. Prospective evaluation of commercial antibody-based rapid tests in combination with a loop-mediated isothermal amplification PCR assay for detection of *Orientia tsutsugamushi* during the acute phase of scrub typhus infection. *Clin Vaccine Immunol 19:* 391–395.
6. Blacksell SD, Bryant NJ, Paris DH, Doust JA, Sakoda Y, Day NP, 2007. Scrub typhus serologic testing with the indirect immunofluorescence method as a diagnostic gold standard: a lack of consensus leads to a lot of confusion. *Clin Infect Dis 44:* 391–401.
7. Luksameetanasan R, Blacksell SD, Kalambaheti T, Wuthiekanun V, Chierakul W, Chueasuwanchai S, Apiwattanaporn A, Stenos J, Graves S, Peacock SJ, Day NP, 2007. Patient and sample-related factors that effect the success of in vitro isolation of *Orientia tsutsugamushi*. *Southeast Asian J Trop Med Public Health 38:* 91–96.
8. Kim YJ, Yeo SJ, Park SJ, Woo YJ, Kim MW, Kim SH, Chang IA, Jeon SH, Park BJ, Song GJ, Lee MG, Kim IS, Kim YW, 2013. Improvement of the diagnostic sensitivity of scrub typhus using a mixture of recombinant antigens derived from *Orientia tsutsugamushi* serotypes. *J Korean Med Sci 28:* 672–679.
9. Blacksell SD, Jenjaroen K, Phetsouvanh R, Wuthiekanun V, Day NP, Newton PN, Ching WM, 2009. Accuracy of AccessBio immunoglobulin M and total antibody rapid immunochromatographic assays for the diagnosis of acute scrub typhus infection. *Clin Vaccine Immunol 17:* 263–266.
10. Blacksell SD, Jenjaroen K, Phetsouvanh R, Tanganuchitcharnchai A, Phouminh P, Phongmany S, Day NP, Newton PN, 2010. Accuracy of rapid IgM-based immunochromatographic and immunoblot assays for diagnosis of acute scrub typhus and murine typhus infections in Laos. *Am J Trop Med Hyg 83:* 365–369.
11. Silpasakorn S, Srisamut N, Ekpo P, Zhang Z, Chao CC, Ching WM, Suputtamongkol Y, 2012. Development of new, broadly reactive, rapid IgG and IgM lateral flow assays for diagnosis of scrub typhus. *Am J Trop Med Hyg 87:* 148–152.
12. Jiang J, Chan TC, Temenak JJ, Dasch GA, Ching WM, Richards AL, 2004. Development of a quantitative real-time polymerase chain reaction assay specific for *Orientia tsutsugamushi*. *Am J Trop Med Hyg 70:* 351–356.
13. Seed P, 2001. *DIAGT: Stata Module to Report Summary Statistics for Diagnostic Tests Compared to True Disease Status*. Boston, MA: Boston College Department of Economics.
14. Landis JR, Koch GG, 1977. The measurement of observer agreement for categorical data. *Biometrics 33:* 159–174.

15. Bourgeois AL, Olson JG, Fang RC, Huang J, Wang CL, Chow L, Bechthold D, Dennis DT, Coolbaugh JC, Weiss E, 1982. Humoral and cellular responses in scrub typhus patients reflecting primary infection and reinfection with *Rickettsia tsutsugamushi*. *Am J Trop Med Hyg 31:* 532–540.
16. Lee SH, Kim DM, Cho YS, Yoon SH, Shim SK, 2006. Usefulness of eschar PCR for diagnosis of scrub typhus. *J Clin Microbiol 44:* 1169–1171.
17. Koh GC, Maude RJ, Paris DH, Newton PN, Blacksell SD, 2010. Diagnosis of scrub typhus. *Am J Trop Med Hyg 82:* 368–370.
18. Watthanaworawit W, Turner P, Turner C, Tanganuchitcharnchai A, Richards AL, Bourzac KM, Blacksell SD, Nosten F, 2013. A prospective evaluation of real-time PCR assays for the detection of *Orientia tsutsugamushi* and *Rickettsia* spp. for early diagnosis of rickettsial infections during the acute phase of undifferentiated febrile illness. *Am J Trop Med Hyg 89:* 308–310.